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14. ABSTRACT

We have shown that the Sonic hedgehog (Shh) signaling pathway is absolutely required for normal prostate development and our studies performed with the support of the DOD New Investigator award indicate that Shh signaling promotes tumor growth. This proposal addresses the hypothesis that Sonic hedgehog signaling promotes tumor growth by activating stromal cell gene expression. To address this hypothesis, we have developed the DLPlacZ-LNCaP xenograft, a model that provides us with the opportunity to selectively assay gene expression in the stromal and epithelial compartments of the tumor using species specific PCR primers and to make specific modifications in stromal cell gene expression. We will use this model to: (1) determine whether Shh promotes tumor growth by activating expression of Gli-1 in the tumor stromal cells; (2) characterize the mechanisms by which tumor growth is promoted; and (3) examine the action of a specific stromal Shh target genes in tumor growth.

15. SUBJECT TERMS
LNCaP, Prostate Cancer, Xenograft, Stroma, Sonic Hedgehog

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INTRODUCTION:

To address the hypothesis that Sonic hedgehog signaling promotes tumor growth by activating stromal cell gene expression, we planned to use a xenograft tumor model in which LNCaP cells are co-injected with cloned, immortalized lacZ expressing stromal cells. The value of this tumor model is that it would provides us with the opportunity to selectively assay gene expression in the stromal and epithelial compartments of the tumor using species specific PCR primers and to make specific modifications in stromal cell gene expression. We planned to use this model to: (1) determine whether Shh promotes tumor growth by activating expression of Gli1 in tumor stromal cells; (2) characterize the mechanisms by which tumor growth is promoted; and (3) examine the action of specific stromal Shh target genes in tumor growth.

BODY:

Studies performed before this proposal was funded compared xenograft tumors made with co-injection of stromal cells from the urogenital sinus or from the different lobes of the adult mouse prostate. Based on those studies, we elected to use a mesenchymal cell line in the experiments proposed. A paper describing the isolation and characterization of the UGSM-2 cell line was published in The Prostate this year. A copy of that paper is attached.

As proposed in Specific Aim1, we have characterized the growth and androgen dependence of the LNCaP-UGSM2 xenograft and shown that Shh overexpression increases growth of the xenograft tumor (Figure 1).

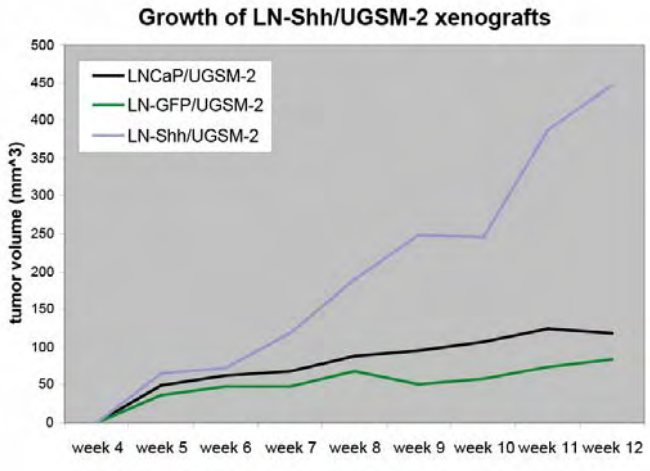


Figure 1. Shh-induced growth of LNCaP-UGSM2 tumors.

To verify that paracrine Hh signaling underlies the Shh growth effect, we analyzed the ability of Shh to induce growth of parent LNCaP in tumors composed of an equal mixture of parent LNCaP and LN-Shh cells. The tumors composed of a mixture of parent LNCaP and LN-Shh cells grew at a faster rate than parent LNCaP tumors, validating the Shh effect in these tumors. Surprisingly, tumors generated by injecting a mixture of LNCaP and LN-Shh cells were composed primarily of parent LNCaP cells after 8 weeks of growth in vivo, confirming that Shh does not have a specific effect on LN-Shh cells, but rather induces paracrine factors that induce growth of parent LNCaP (Figure 2).

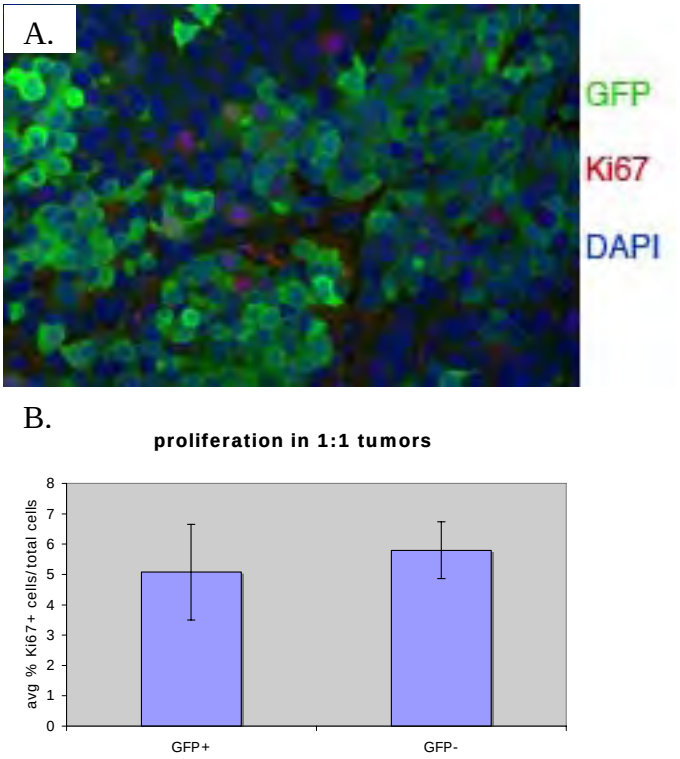
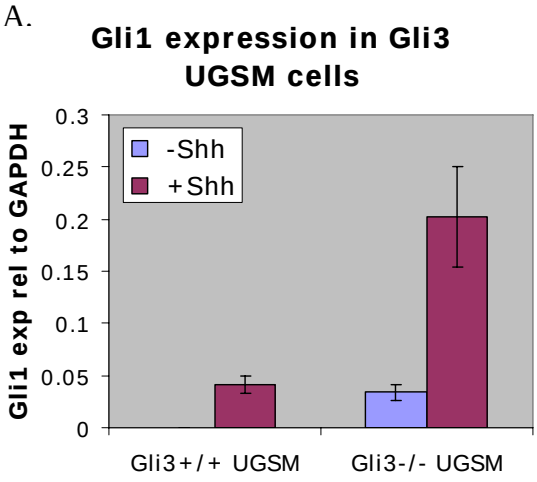


Figure 2. Shh signaling induces paracrine signaling that stimulates growth of LNCaP. (A) immunohistochemical staining for GFP and Ki67 shows that parent LNCaP and LN-Shh-GFP cells grow equally in 1:1 mix tumors. (B) Proliferation rates of GFP+ LN-Shh and GFP- parent LNCaP cells in 1:1 mix tumors. LNCaP and LN-Shh cells proliferate at the same rate, indicating that Shh does not induce cell proliferation in a cell autonomous manner.

To determine if stromal Hh signaling activation mimics the Shh growth effect, we generated a stromal cell line that lacks Gli3, a repressor of Hh signaling. Gli3 null stromal cells exhibit active Hh signaling in the absence of Shh. Co-injection of Gli3 null stromal cells resulted in rapid growth of tumors when compared with tumors containing wild-type stromal cells (Figure 3). A paper describing these studies is being prepared for submission.



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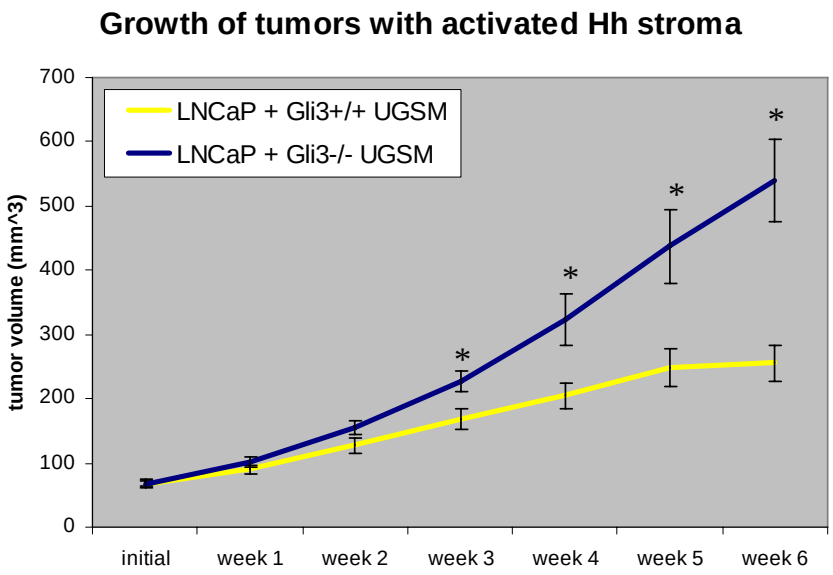


Figure 3. Stromal Hh signaling induces rapid tumor growth. (A) Gli3-/- cells have activated Hh signaling in the absence of Hh ligand. (B) Tumors containing Gli3-/- stromal cells grow faster than tumors containing wild-type stromal cells.

Detailed analysis of Hh signaling in LNCaP revealed that LNCaP do not respond to Shh by increasing expression of the canonical Hh signaling mediators Gli1 and Ptc1 (Figure 4). In fact, expression of Smo in 22RV1 or PC-3 cells does not induce pathway activation as it does in other cell lines (Figure 5). However, expression of Gli1 or Gli2 in 22RV1 or PC-3 cells does induce transcription of Hh target genes Gli1 and Ptc (Figure 6). These studies revealed that intracellular Hh signal transduction in LNCaP is functionally impaired and pathway target genes can only be induced by expression of the final mediators of the Hh transcriptional response. We have recently found that Hh signaling is similarly impaired in LNCaP (not shown), lending credence to the idea that LNCaP are not capable of Hh signal transduction and growth effects on tumors must be mediated by paracrine interactions with other cells in the tumor.

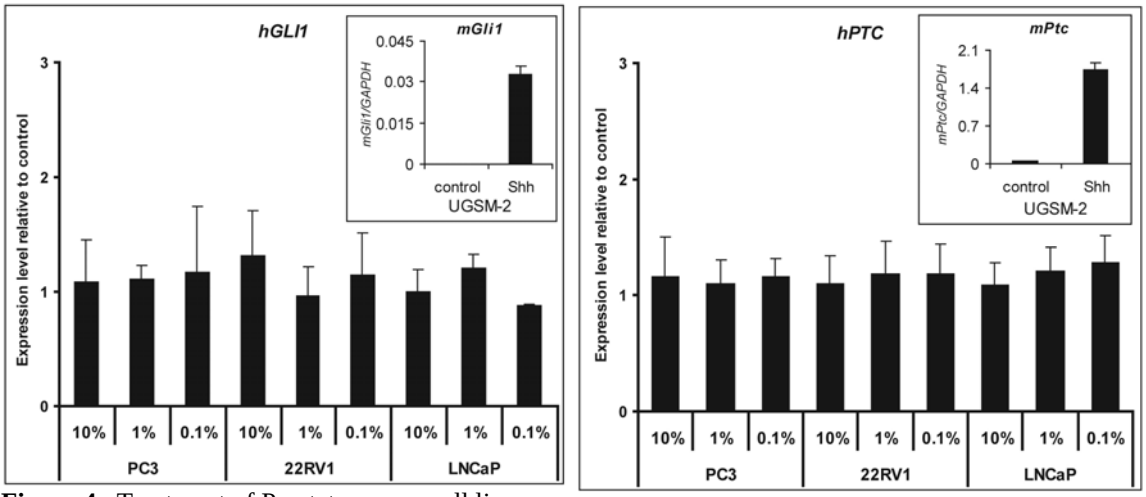


Figure 4. Treatment of Prostate cancer cell lines with 1 nM purified Shh peptide does not induce expression of Hh target genes Ptc and Gli1. (inset) Treatment of UGSM-2 mesenchymal cells with the same dose of Shh causes a ~100-fold increase in expression of Hh target genes Ptc and Gli1.

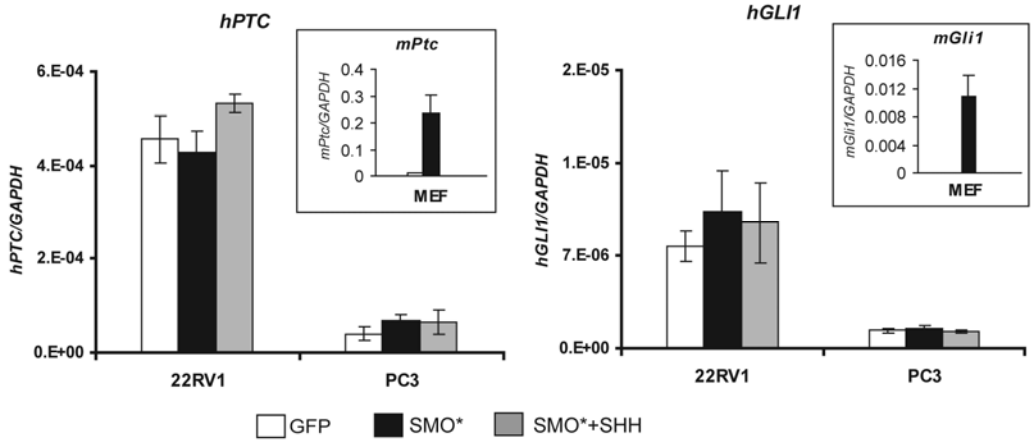


Figure 5. Expression of constitutively active Smo, an inducer of Hh signaling, fails to induce expression of Hh target genes in 22RV1 and PC-3 human prostate cancer cell lines, but the same expression construct faithfully induces Hh signaling in mouse embryonic fibroblasts (inset).

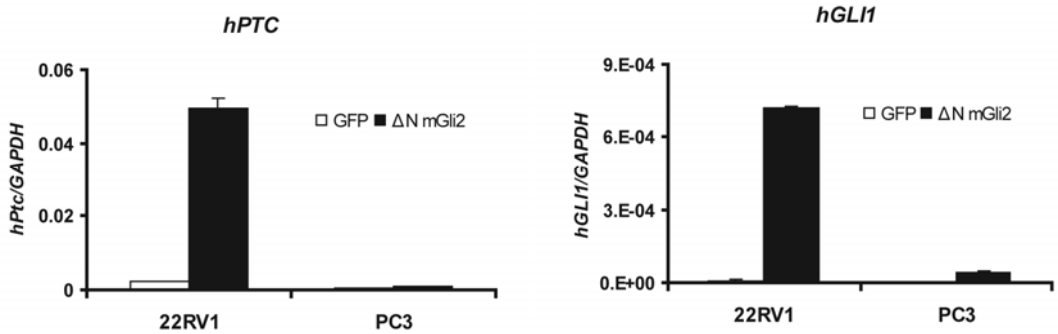


Figure 6. Expression of constitutively active Gli2 in 22RV1 or PC-3 cells induces Hh signaling.

Collectively, these results indicate that Shh expression by LNCaP in LN-Shh tumors induces stromal Hh signaling that leads to accelerated tumor growth. The paper describing these studies was recently published along with a review of Hh signaling in the prostate. Those papers are attached.

To explain the absence of Hh responsiveness in cultured cell lines, we examined the signal transduction apparatus in PC3, LNCaP and 22RV1. These studies revealed the absence of cilia in these cell lines. Since cilia is necessary for Hh signal transduction, this explains their lack of response to Hh ligand. In contrast, we have shown that Hh responsiveness of the UGSM-2 cell line correlates with the appearance of cilia and, further, that Shh induces Smo movement to the cilia as part of the signal transduction process. These studies were not part of this funded proposal and therefore the data is not presented in detail, but the observations are summarized here since the project was a direct offshoot of the funded work. A manuscript describing these studies is in preparation.

As proposed in Specific Aim2, we have identified new Hh target genes in stromal cells and have begun correlating expression of these genes with accelerated tumor growth in LN-Shh tumors. We have identified 40 new target genes and are currently in the process of determining if these genes are modified in LN-Shh tumors with accelerated growth. On the basis of its known role in prostate development, we analyzed expression of Noggin in LN-Shh vs. parent LNCaP tumors. Noggin expression is significantly elevated in LN-Shh tumor stroma (Figure 7).

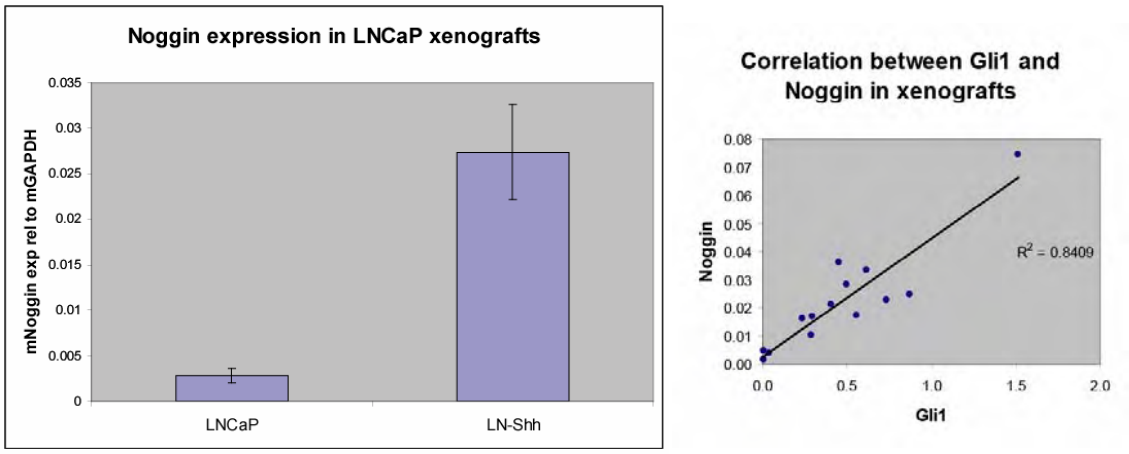


Figure 7. Noggin expression is increased in stroma of LN-Shh tumors and correlates with stromal Gli1 expression.

Noggin is a secreted BMP antagonist and blocks BMP inhibition of LNCaP proliferation in vitro (Figure 8). BMP signaling correlates with expression of Id-1 in LNCaP and LN-Shh tumors exhibit decreased BMP signaling as a result of Noggin overrexpression in the stroma (Figure 9). A paper describing this work is being readied for submission.

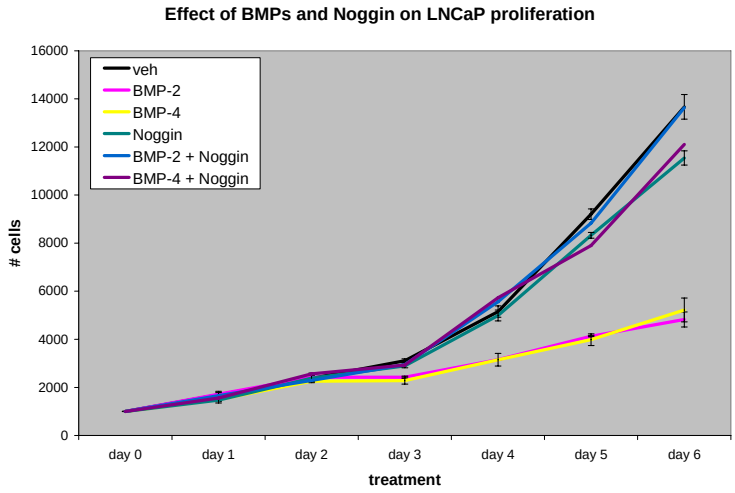


Figure 8. Growth of LNCaP in culture was measured daily after treatment with recombinant proteins. BMP-2 and BMP-4 inhibited cell growth while Noggin had no effect. However treatment with BMP together with Noggin reversed the BMP inhibitory effect.

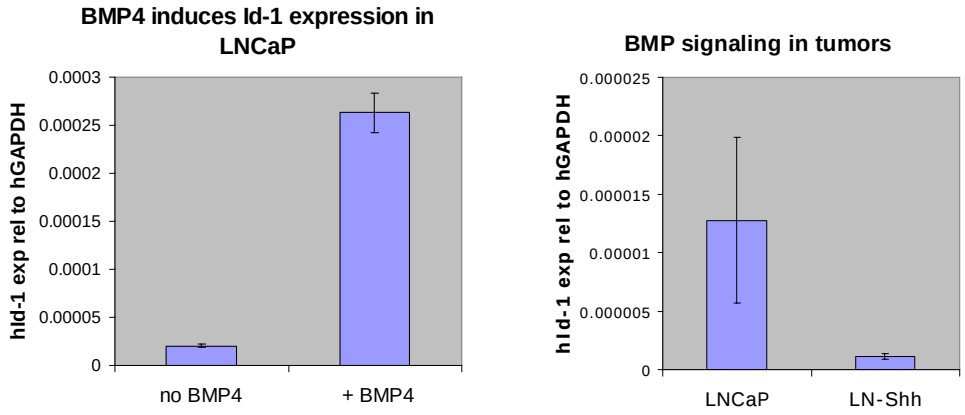


Figure 9. Reduced BMP signaling in LN-Shh tumors. BMP4 treatment in vitro induces expression of Id-1 in LNCaP. LN-Shh tumors that express high levels of the BMP antagonist Noggin have reduced BMP signaling, as evidenced by Id-1 expression.

We have identified Shh target genes in LNShh tumor stroma. UGSM-2 cells were treated with Shh ligand for 24 hours and target genes were identified by microarray. The microarray experiment revealed that 90 genes are modified. We used qRT-PCR and blockade by the Hh antagonist cyclopamine to verify 17 Shh target genes in vitro. These genes are listed in Table 1. Analysis of the 17 target genes tumors revealed that 8 of these genes are modified in the LN-Shh tumor stroma. Of these 8 genes, 3 of these genes are upregulated in Gli3-/- UGSM cells. This data is listed in Table 2.

	UGSM-2 Cells		
Gene	Array w/ Shh	Validation w/ Shh	w/ Shh& Cyclopamine
Gli1	17.1	621	2.31
Ptc1	8.6	34	0.74
BRAK	27.6	18.4	0.48
Dner	24.9	15.87	0.26
Fgf5	9.1	22.56	1.56
Map3k12	3.97	0.96	1.06
Timp3	3.81	3.26	0.45
Angpt4	3.51	9.44	0.91
Hsd11b1	3.48	12.58	0.48
Tnmd	3.34	2.58	0.62
Artn	2.67	1.45	0.59
Fbn2	2.55	3.08	0.63
Igfbp-3	2.51	3.02	0.24
Ntrk3	2.4	0.9	0.95
Tiam1	2.3	3.16	0.18
Igfbp-6	2.28	2.83	1.31
Sod3	2.26	3.5	0.46
Plxna2	2.2	2.69	0.86
Sos1	2.04	0.9	0.9
Inhbb	1.03	2.08	0.84
Spdy	0.5	1.01	0.39
Rgs4	0.44	0.49	0.6
Fkbp1a	0.41	1.14	0.97
Sufu	0.37	0.97	0.64
Mmp13	0.21	0.28	0.23
Dmp1	0.16	0.4	2.42

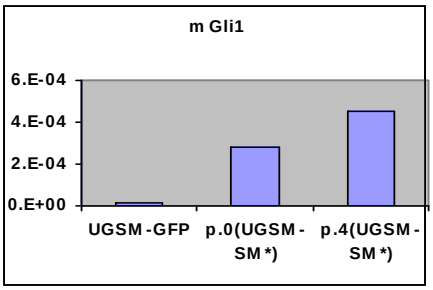
Table 1. Shh target genes were analyzed by microarray analysis, RT-PCR validation and inhibited with cyclopamine. We identified 24 targets by array, validated 17 by RT-PCR and 16 of these are inhibited by cyclopamine.

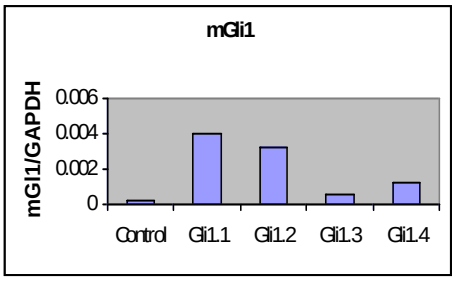
Gene	LNShh tumors	Gli3-/- cells
Gli1	increase	increase
Ptc1	increase	increase
Angpt4	increase	decrease
Artn	no change	ND
BRAK	increase	no change
Dmp1	no change	ND
Dner	no change	increase
Fbn2	increase	ND
Fgf5	increase	no change
Hes1	increase	increase
Hsd11b1	increase	decrease
Igfbp-3	no change	ND
Igfbp-6	increase	increase
Ntrk3	no change	ND
Plxna2	no change	ND
Sod3	no change	ND
Tiam1	no change	ND
Timp3	increase	increase
Tnmd	no change	ND

Table 2. 17 Shh target genes and 2 canonical genes were analyzed in LNShh tumor stroma and in UGSM-Gli3 cells. Data illustrated shows if genes are changed in LNShh tumors relative to LNCaP tumors (middle column) or are changed in UGSM-Gli3-/- cells relative to UGSM-Gli3+/+ cells in culture (last column).

To perform the studies described in Specific Aim 3, we have generated UGSM-2 cells which overexpress Smo, Gli1 or Gli2 and UGSM-2 cells (Figure 10). Over-expression of Gli1 did not accelerate tumor growth, but the interpretation is limited by the inability to achieve robust over-expression in vivo (Figure 11 and 12).

Attempts to increase Gli2 over-expressing stromal cells were unsuccessful because the over-expressing cells formed sarcomas in vivo. Our attempts to use a newly identified dominant negative Gli2 mutant to abrogate Hh signaling in the stromal cells was unsuccessful because cells transfected with the Gli2 construct were not viable (data not shown).





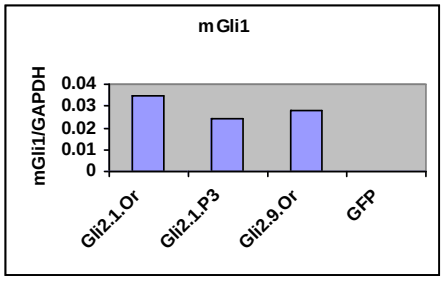


Figure 10. UGSM-2 cells were treated with human SmoM2 (top), Gli1 (middle) or Gli2 (bottom) retrovirus. Shh signaling in different passages of cells was determined by examining mouse Gli1 expression by RT-PCR. Robust induction of Hh signaling was maintained by overexpression of SmoM2 and Gli2, but not by Gli1.

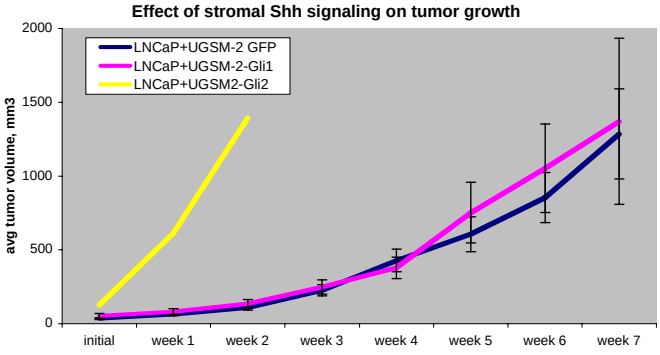


Figure 11. LNCaP were co-injected with UGSM2-SmoM2.Gli1/Gli2 cells. Tumor growth was measured as is illustrated as the average for each tumor type. LNCaP + UGSM2-Gli2 tumors formed rapidly and mice had to be sacrificed early.

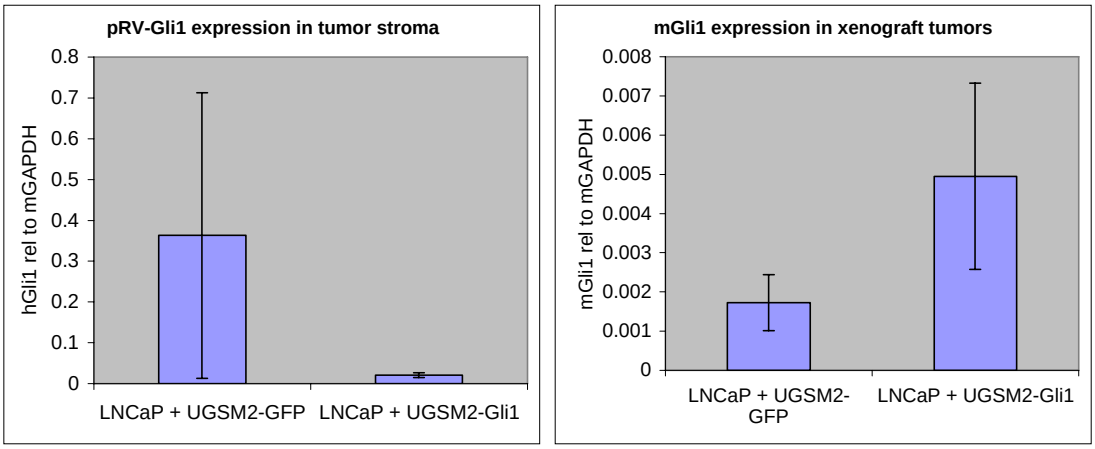


Figure 12. Gene expression in LNCaP + UGSM2-GFP/Gli1 tumors. Expression of retroviral human Gli1 is not significantly increased in UGSM2-Gli1 tumor stroma. There is a small increase in mouse Gli1 in UGSM2-Gli1 tumor stroma, but is not significant ($p = 0.143$).

The second portion of Specific Aim 3 is to examine the effect of stromal Hh target expression on tumor growth. To this end, we have stably overexpressed Noggin in UGSM-2 stromal cells and generated tumors by combining LNCaP + UGSM2-Noggin cells. Analysis of the growth of these tumors showed that Noggin overexpression in tumor stroma does not accelerate tumor growth, nor is Noggin overexpression additive with Shh for inducing tumor growth (Figure 13). Thus, Noggin is not sufficient to stimulate tumor growth. A manuscript describing this work, together with the studies on Noggin described above, is being readied for submission.

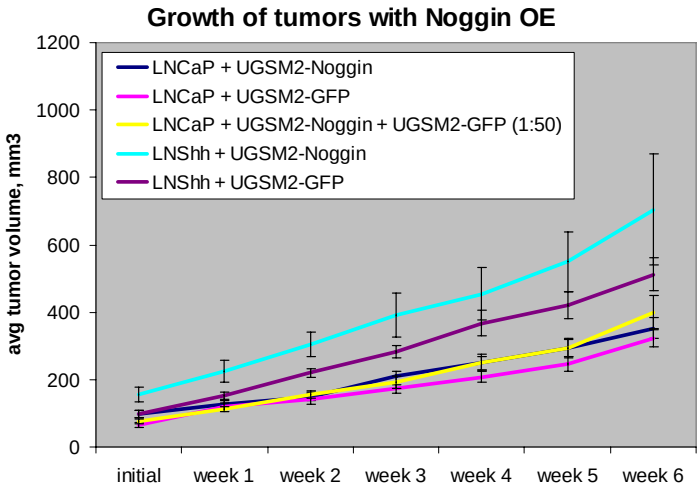


Figure 13. Noggin over-expression in tumor stroma does not stimulate tumor growth. Stromal Noggin overexpression does not induce tumor growth in addition to Shh.

KEY RESEARCH ACCOMPLISHMENTS:

We have developed and characterized a new mesenchymal cell line that will be used to genetically manipulate gene expression in tumor cells, tumor stromal cells or both.

We have confirmed that Shh-induced rapid tumor growth also occurs in bi-clonal xenograft tumors created by co-injecting LNCaP cells with UGSM-2 cells.

We have shown that paracrine Hh signaling is the primary mechanism of Shh-induced tumor growth.

We have generated a set of retroviral expression constructs containing Shh pathway genes for use in determining the role of each of these genes in Shh accelerated tumor growth. We have generated UGSM and LNCaP cell lines stably expressing these genes.

We have generated stromal cell lines lacking Gli1 and Gli2 for the purpose of identifying stromal Gli1 and Gli2 roles in Shh-induced tumor growth.

We have identified Noggin as a gene overexpressed in stroma of LN-Shh tumors. Stromal Noggin overexpression alone is not sufficient to accelerate tumor growth.

REPORTABLE OUTCOMES:

A manuscript which describes the isolation and characterization of the UGSM-2 cell line was published in Prostate (manuscript attached).
A manuscript examining hedgehog signaling in human prostate cancer cell lines was published in the Journal of Urology (manuscript attached).
A review of hedgehog signaling in prostate cancer published the Journal of Urology (manuscript attached).

Invited Speaker – SBUR December, 2005 Miami Beach, Florida

SBUR Keynote Speaker Award, 2005 Miami Beach, Florida

CONCLUSIONS:

Three additional manucripts are being readied for submission.

REFERENCES:

None

APPENDIX:

Manuscripts attached

The Prostate 66:1347–1358 (2006)

Isolation and Characterization of an Immortalized Mouse Urogenital Sinus Mesenchyme Cell Line

Aubie Shaw,[1] John Papadopoulos,[2] Curtis Johnson,[2] and Wade Bushman[2]*

[1]McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin
[2]Department of Surgery, University of Wisconsin, Madison, Wisconsin

BACKGROUND. Stromal-epithelial signaling plays an important role in prostate development and cancer progression. Study of these interactions will be facilitated by the use of suitable prostate cell lines in appropriate model systems.
METHODS. We have isolated an immortalized prostate mesenchymal cell line from the mouse E16 urogenital sinus (UGS). We characterized its expression of stromal differentiation markers, response to androgen stimulation, ability to induce and participate in prostate morphogenesis, response to Shh stimulation, and interaction with prostate epithelial cells.
RESULTS. UGSM-2 cells express vimentin and smooth muscle actin, but not the mature smooth muscle markers myosin and desmin. This expression profile is consistent with a myofibroblast phenotype. Unlike other fibroblasts such as 3T3, UGSM-2 cells express androgen receptor mRNA and androgen stimulation increases proliferation. UGSM-2 cells are viable when grafted with embryonic UGS under the renal capsule and participate in glandular morphogenesis, but are not capable of inducing prostate morphogenesis of isolated UGS epithelium. Co-culture of UGSM-2 cells with human BPH-1 cells or co-grafting in vivo results in organized clusters of BPH-1 cells surrounded by a mantle of UGSM-2 cells. UGSM-2 cells are responsive to Sonic hedgehog (Shh), an important signaling factor in prostate development, and mimic the transcriptional response of the intact UGS mesenchyme. In co-cultures with BPH-1, UGSM-2 cells exhibit a robust transcriptional response to Shh secreted by BPH-1.
CONCLUSIONS. UGSM-2 is a urogenital sinus mesenchyme cell line that can be used to study stromal-epithelial interactions that are important in prostate biology. *Prostate 66: 1347–1358, 2006.* © 2006 Wiley-Liss, Inc.

KEY WORDS: **stromal-epithelial interactions; androgen; sonic Hedgehog; prostate development; mesenchyme**

INTRODUCTION

The prostate develops from a specific region of the endodermal urogenital sinus (UGS) termed the prostatic anlagen. Formation of the prostatic ducts begins at embryonic day 17 (E17) in the mouse when epithelial buds evaginate into the surrounding mesenchymal sheath. Discrete groups of buds define the origins of the anterior, dorsal and ventral lobes of the prostate. At the time of ductal budding, the UGS mesenchyme is composed of undifferentiated fibroblasts and myofibroblasts. As the buds elongate, they lumenalize to form true secretory ducts connected to the urethral lumen and branch to form a highly complex ductal tree. As the ducts grow, they are surrounded by a sheath of mesenchyme, which differentiates to a periductal stroma comprised of smooth muscle cells and fibroblasts [1].

The embryonic mesenchyme and its adult descendent stroma have emerged as key regulators of prostatic growth and differentiation. In the UGS, mesenchymal cells express androgen receptors and act under the influence of androgens to induce prostatic differentiation of the endodermal epithelium [2,3].

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Tissue recombination experiments have shown that the mesenchyme is the primary determinant of epithelial growth and differentiation [4]. In the adult prostate, there is regional heterogeneity within the ducts: the distal tips are encased in a delicate fibroblastic sheath, while the more proximal segments are surrounded by thicker sheaths rich in smooth muscle [5]. Androgen receptor expression is localized to the dense smooth muscle sheath surrounding epithelial ducts, whereas fibroblasts rarely express androgen receptors [6]. Smooth muscle is required for maintenance of epithelial secretory function [7] and loss of smooth muscle in the adult prostate is associated with cancer lesions and de-differentiation of epithelium [8].

Primary stromal cells from human prostate tissue have been used to discover factors that regulate smooth muscle differentiation and proliferation of prostate stroma, and to identify stromal-derived factors that regulate epithelial functions. Several prostate stromal cell lines have been generated, including rat NbF-I, mouse PSMC1, rat PS-1, human WPMY-1, human DuK50, and human PS30 cells [9–14]. Two rat UGS mesenchymal cell lines have been generated: rUGM and U4F1 [15,16]. To our knowledge, none of these cell lines is able to induce or participate in prostate morphogenesis.

The signaling interactions that regulate prostate ductal budding and branching morphogenesis have received considerable attention as the paradigm for understanding normal prostate growth regulation. These studies have demonstrated that the UGS mesenchyme is the target of several key signals, including testosterone, estrogen, and sonic hedgehog [17–19]. UGS mesenchyme is also the origin of several key morphogens including BMP-4, FGF-10, TGFβ [20–23], and Shh target genes such as IGFBP-6 [31], which may regulate both epithelial and mesenchymal proliferation and differentiation. The complexity of these interactions is daunting. For the Shh pathway alone, there are three different Gli genes expressed in the UGS mesencyhme and each of these plays a unique role in the transcriptional response to Hh signaling [18]. Similar complexities exist in the multiplicity of receptor subtypes for BMP, TGFβ, and FGF signaling. To elucidate the complex regulation and crosstalk between these pathways in mesenchymal cells, we have developed an immortalized UGS mesenchymal cell line and demonstrated that it phenocopies the UGS mesenchyme response to Shh stimulation.

Several unique characteristics distinguish the mesenchyme of the urogenital sinus. These include responsiveness to androgen, the ability to induce prostate differentiation of isolated urogenital sinus epithelium, and responsiveness to morphogens such as Sonic hedgehog. UGSM-2 cells were found to be androgen responsive and to mimic the canonical response of urogenital sinus mesenchyme to Sonic Hedgehog. UGSM-2 cells did not induce morphogenesis of isolated UGS epithelium sheets, but when grafted together with the E16 UGS they did proliferate and become incorporated into the periductal stroma during glandular morphogenesis.

MATERIALS AND METHODS

Animals and Cell Lines

Balb/c 3T3 fibroblasts were obtained from ATCC and cultured according to ATCC guidelines. BPH-1 cells were obtained from Simon Hayward (Vanderbilt University, Nashville, TN) and maintained in RPMI + 25 mM HEPES + 10% FBS. UGSM-2 cells were maintained in DMEM/F12 + ITS + 10% FBS + 10^{-8} M DHT. Wild-type CD-1 and CD-1 nude mice were obtained from Charles River (Wilmington, MA). INK4a$^{-/-}$, β-actin-tva transgenic mice were obtained from Bart Williams (Van Andel Research Inst., Grand Rapids, MI). All animals were housed according to institutional animal use and care guidelines.

Isolation of UGSM-2 Cells

Immortalized UGSM-2 cells were derived from the urogenital sinus of an E16 male INK4a$^{-/-}$ tva transgenic mouse embryo. INK4a$^{-/-}$, β-actin-tva transgenic mice were provided by Bart Williams (Van Andel Research Institute, Grand Rapids, MI). UGS epithelium was separated from mesenchyme following trypsin digestion as described previously [24]. Mesenchyme was further dissociated into single cells by digestion in 0.5% collagenase. Dissociated mesenchymal cells were grown in DMEM + 15% FBS + 1% pen/strep until they reached confluence in a 6-well plate. Thereafter cells were grown in DMEM/F12 + 10% FBS + 1% pen/strep + 1% ITS + 10^{-8} M DHT (INK4 culture medium). The UGSM-2 clone was isolated from the mixed UGSM population by dilution cloning followed by ring cloning.

Growth Curve Analysis

UGSM-2 and 3T3 cells were plated at a density of 4×10^4 cells per well in 6-well plates in normal culture media containing 10% charcoal-stripped, dextran-treated fetal bovine serum, csFBS (Hyclone, Logan, UT). After 48 hr, cells were treated with 10^{-8} M R1881 or 0.1% ethanol in normal culture media containing 10% csFBS. Each day, cells were trypsinized, diluted 1:100 in Isoton II solution (Beckman, Fullerton, CA) and counted in triplicate using a ViCell XR viable cell counter (Beckman Coulter). No significant difference in

cell viabilities between treatments was noted. Doubling time was calculated by determining the time required to double the number of cells in linear mid-log phase.

Ploidy Analysis

UGSM-2 cells were determined to be tetraploid by comparison to ploidy number of known diploid cells: freshly isolated splenocytes from the spleen of a CD-1 mouse (Charles River, Wilmington, MA). Splenocytes and UGSM-2 cells were combined in the following three ways: (1) 2×10^6 splenocytes, (2) 0.5×10^6 splenocytes $+ 2 \times 10^6$ UGSM-2 cells, and (3) 2×10^6 UGSM-2 cells. Cells were pelleted and fixed in ice cold 70% ethanol for 30 min. Cells were then pelleted and resuspended in 33 μg/ml propidium iodide + 1 mg/ml RNase A + 0.2% Nonidet P-40 in PBS. DNA content of cells was determined using a FACScan cytometer and analyzed using ModFitLT V3.0 software.

Immunocytochemistry

UGSM-2 cells were grown on Lab-Tek II chamber slides (Fisher, Pittsburgh, PA) and immunostained for vimentin, smooth muscle actin (SMA) or pan-cytokeratin (pan-CK). Slides were fixed in 4% paraformaldehyde and blocked in 5% normal goat serum in PBS. Anti-vimentin clone LN-6 (Sigma, St. Louis, MO), anti-smooth muscle actin monoclonal antibody clone 1A4 (Sigma) or anti-pan-cytokeratin monoclonal antibody (Zymed, South San Francisco, CA) was applied at a dilution of 1:200. Staining was visualized by incubating with goat anti-mouse Alexa 546 conjugated antibody (Molecular Probes, Eugene, OR) at a dilution of 1:200. Slides were mounted with Vectashield Hardset + DAPI mounting media (Vector, Burlingame, CA) and imaged using an Olympus model BX51 fluorescent microscope and Spot Advanced software v. 3.5.2.

RT-PCR

RNA was isolated from confluent cells using RNeasy mini kit (Qiagen, Valencia, CA) with optional on-column DNase digestion to eliminate contaminating DNA. Total RNA (1 μg) was reverse transcribed to generate cDNA using M-MLV reverse transcriptase (Invitrogen). Relative mRNA quantity was determined by real-time RT-PCR using iCycler instrumentation and software (BioRad, Hercules, CA). Primer sequences are listed in Table I. Primer sets whose name starts with 'm' are mouse-specific, while primer sets whose name starts with 'h' are human-specific. All sequences are listed in 5′–3′ orientation.

Co-Cultures

UGSM-2 and BPH-1 cells were plated at equal densities (1×10^6 cells each) in 25 cm^2 flasks coated with neutralized rat tail collagen [25]. Morphology of cells was observed and photographed over a 1-week period using a Nikon Eclipse TS100 inverted light microscope with a Spot Insight QE digital camera. RNA was prepared from 48 hr co-cultures as described above. Expression of Shh signaling targets Gli1 and Ptc1 was examined by RT-PCR.

Renal Capsule Grafts

For UGE + UGSM-2 grafts, E16 UGSs were separated into epithelium (UGE) and mesenchyme (UGM) using the method described previously [24]. UGE + UGSM-2 were combined and allowed to adhere together overnight on 0.6% agar plates containing INK4 culture medium. For UGS+UGSM-2 grafts, UGSs were dissected from E16 male CD-1 mouse embryos and chopped into five to six pieces, combined with UGSM-2 cells, and incubated overnight on agar plates prepared

TABLE I. Sequences of RT-PCR Primers

Target gene	Forward primer	Reverse primer
mGAPDH	AGCCTCGTCCCGTAGACAAAAT	CCGTGAGTGGAGTCATACTGGA
mSMA	ATCATGCGTCTGGACTTGG	AATAGCCACGCTCAGTCAGG
mVim	CCCCCTTCCTCACTTCTTTC	AAGAGTGGCAGAGGACTGGA
mDesmin	GTGAAGATGGCCTTGGATGT	TTGAGAGCAGAGAAGGTCTGG
mHCM	GCAGCTTCTACAGGCAAACC	CAAAGCGAGAGGAGTTGTCG
mAR	GTGAAGCAGGTAGCTCTGGG	GAGCCAGCGGAAAGTTGTAG
mCD31	CTGAGCCTAGTGTGGAAGCC	TACATCCATGTTCTGGGGGT
mGli1	GGAAGTCCTATTCACGCCTTGA	CAACCTTCTTGCTCACACATGTAAG
mPtc1	CTCTGGAGCAGATTTCCAAGG	TGCCGCAGTTCTTTTGAATG
mIGFBP6	AGCTCCAGACTGAGGTCTTCC	GAACGACACTGCTGCTTGC
mHIP	CCTGTCGAGGCTACTTTTCG	TCCATTGTGAGTCTGGGTCA
hGAPDH	CCACATCGCTCAGACACCAT	GCAACAATATCCACTTTACCAGAGTTAA
hGli1	AATGCTGCCATGGATGCTAGA	GAGTATCAGTAGGTGGGAAGTCCATAT
hPtc1	CGCTGGGACTGCTCCAAGT	GAGTTGTTGCAGCGTTAAAGGAA

with INK4 culture media. For BPH-1 + UGSM-2 grafts, 500,000 UGSM-2 and 100,000 BPH-1 cells were resuspended in cold Matrigel (BD Biosciences, Becton, MA) and allowed to gel in sterile culture dishes. After 30 min, Matrigel beads containing cells were covered with INK4 culture medium and placed in a CO_2 incubator overnight. Recombinants were placed under the renal capsule of CD-1 adult male nude mice using the method outlined by Cunha et al. (http://mammary.nih.gov/tools/mousework/Cunha001/Pages/Navigation.html). After 1–4 weeks, grafts were harvested, fixed, and paraffin-embedded sections were prepared.

BrdU Pulse and Immunolabeling

BrdU labeling was used to trace UGSM-2 cells in renal grafts. Subconfluent UGSM-2 cells were incubated with 10 μM bromodeoxyuridine (BrdU) in normal culture media overnight. Overnight incubation with BrdU resulted in approximately 50% of cells with BrdU incorporated. Immunolabeling of cells in formalin fixed paraffin-embedded sections was accomplished using the BrdU Labeling and Detection Kit II (Roche, Indianapolis, IN). We used goat anti-mouse-Alexa 546 conjugated antibody (Molecular Probes) to visualize BrdU stained cells. Sections were co-stained for pan-cytokeratin (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:50 dilution. Pan-CK was visualized by incubating with goat anti-rabbit Alexa 488 conjugated antibody (Molecular Probes, Eugene, OR) at a dilution of 1:200. Sections were mounted with Vectashield Hardset mounting media + DAPI counterstain (Vector).

Shh Treatment

UGSM-2 cells were plated in 6-well plates at a density of 4×10^5 cells/well in complete media and allowed to attach overnight. The next day, cells were treated with 1 nm octylated N-Shh peptide (Curis, Inc., Cambridge, MA). After 48 hr, cells were lysed and RNA was collected. RNA was purified and prepared for RT-PCR as described above.

Shh Overexpression

A mammalian expression vector expressing human Shh driven by CMV promoter (pIRES2-hShh-EGFP) was constructed as described previously [26]. BPH-1 cells were transfected with pIRES2-hShh-EGFP vector or pIRES2-EGFP vector control (Clontech, Palo Alto, CA) using Lipofectamine 2000 (Invitrogen). BPH-1 cells stably overexpressing Shh/GFP were derived by fluorescence-activated cell sorting for GFP for 2 months after transfection. BPH-Shh cells stably express 50,000-fold more Shh mRNA than BPH-GFP or parent BPH-1 cells.

Statistical Analysis

An unpaired *t*-test was used to determine if significant differences exist between cell growth rates for untreated, testosterone or dihydrotestosterone treated cells. The Wilcoxon Rank Sum test was used to determine if there were significant differences in the gene expression responses to Shh treatment.

RESULTS

Isolation and Characterization of UGSM-2 Cells

Immortalized UGS mesenchymal (UGSM) cells were derived from a subline of the *INK4a* mouse, a transgenic knockout that lacks $p16^{INK4a}$ and $p19^{ARF}$. Both $p16^{INK4a}$ and $p19^{ARF}$ are specific inhibitors of cyclin-dependent kinases Cdk4 and Cdk6 that regulate cell cycle progression [27]. Loss of $p16^{INK4a}$ and $p19^{ARF}$ allows mouse embryonic fibroblasts (MEFs) to escape cellular senescence. $INK4a^{-/-}$ MEFs spontaneously immortalize in culture [28]. UGSM cells were isolated by dissecting UGS mesenchyme from an E16 $INK4a^{-/-}$ mouse embryo (Fig. 1A). UGSM cells obtained in this fashion were propagated continuously without evidence of crisis. Immortalized mouse cells are typically tetraploid and these cells remained stably tetraploid for over 100 passages (data not shown). Several ring clones were derived and characterized. All exhibited a similar growth rate and morphology in culture and all responded to treatment with Sonic Hedgehog by upregulating transcription of the conserved Hh target genes Ptc and Gli1. One representative clonal cell line, UGSM-2, was selected for use in subsequent experiments. Like the parent mixed cell population, UGSM-2 cells were found to be stably tetraploid (Fig. 1B). Recent studies revealed that $INK4a^{-/-}$ MEFs can acquire chromosomal rearrangements at high passage [29]. To assess tumorigenicity, both the parent UGSM cell line and UGSM-2 cells were co-injected with Matrigel into the flanks of nude mice. No tumor formation was observed in any of 12 injections for each group of cells over 6 months observation, whereas co-injection of LNCaP cells with Matrigel at the same time yielded tumor formation at over 80% of sites injected within 6 weeks (data not shown). Sarcoma formation was observed when a mixed population of UGSM cells at high passage (>30) were injected into nude mice, however, we have never observed sarcoma formation with the UGSM-2 clone.

UGSM-2 Cells Display a Myofibroblast Phenotype in Culture

The mesenchymal identity of UGSM-2 cells was established by characterizing expression of selected differentiation markers by RT-PCR and immunocyto-

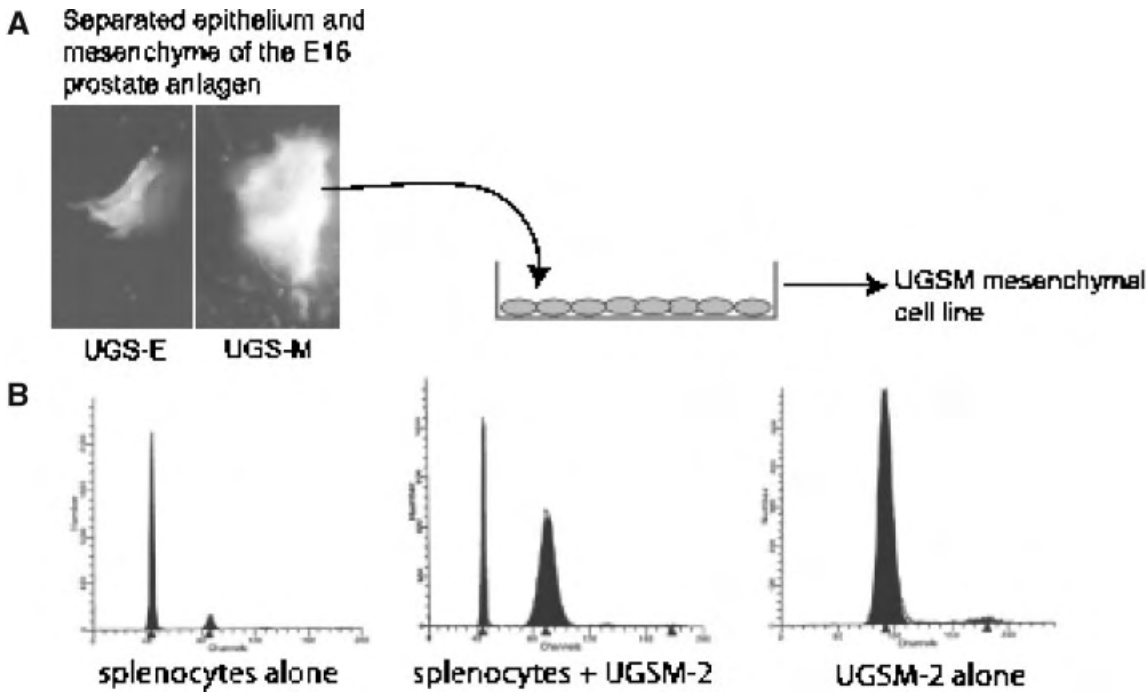

Fig. 1. Isolation of UGSM-2 cells. (**A**) Schematic of UGSM-2 isolation from the E16 UGS mesenchyme of an INK4a−/−; tva transgenic mouse. (**B**) The clonal cell line UGSM-2 is tetraploid on flow cytometric analysis. Freshly isolated mouse splenocytes were used as a diploid comparison.

chemistry (Fig. 2). UGSM-2 cells express the stromal differentiation markers smooth muscle actin (SMA) and vimentin, and do not express either cytokeratins or the endothelial marker CD31/PECAM. The prostatic stroma contains cells that are classified as fibroblasts or smooth muscle, as well as cells termed myofibroblasts, which exhibit an intermediate phenotype. The profile of four stromal markers has been used to characterize cells as fibroblast (SMA−, vimentin+, desmin−, HCM−), myofibroblast (SMA+, vimentin+, desmin−, HCM−), or smooth muscle (SMA+, vimentin−, desmin+, HCM+) [30]. According to this classification UGSM-2 cells, which express SMA and vimentin, but do not express either desmin or heavy chain myosin (HCM) would be considered to exhibit a myofibroblast phenotype.

Growth Characteristics of the UGSM-2 Cell Line

Growth of many cell lines in culture is characterized by three phases: a lag phase while cells attach to the substrate; a log phase of exponential growth; and a plateau phase triggered by confluence and contact inhibition. UGSM-2 cell growth in culture exhibits all three phases of growth. The typical doubling time for UGSM-2 cells in normal culture media is 13 hr (Fig. 3A). The presence of a plateau phase shows that UGSM-2 cells are contact inhibited and, indeed, the cells at confluence adopt a tight monolayer appearance (Fig. 5A).

Androgen Response of UGSM-2 Cells

The fetal urogenital sinus mesenchyme expresses androgen receptor and the androgen response of UGS mesenchyme is an important aspect of prostate biology. We examined androgen receptor expression by RT-PCR and found that UGSM-2 cells express the androgen receptor at levels comparable to the E16 UGS. Another fibroblast cell line that is not derived from the embryonic urogenital sinus, 3T3 fibroblasts, do not express androgen receptor (Fig. 2B). UGSM-2 cells are not dependent on androgen for survival or proliferation (data not shown), however, their proliferation in culture is androgen sensitive. When we compared UGSM-2 growth in charcoal stripped serum supplemented medium without exogenous steroid hormone or with 10^{-8} M synthetic androgen R1881, we found that UGSM-2 cells cultured in the presence of androgen grow at a significantly faster rate. 3T3 fibroblasts do not increase their proliferation rate in response to androgen (Fig. 3B). The same effects were seen with either 10^{-8} M testosterone or dihydrotestosterone (data not shown).

Participation of UGSM-2 in Prostate Morphogenesis

Our goal in developing the UGSM-2 cell line was to create a genetically modifiable cell line that could be used to study specific stromal-epithelial signaling interactions in prostate development. We therefore examined the ability of UGSM-2 cells to mimic three

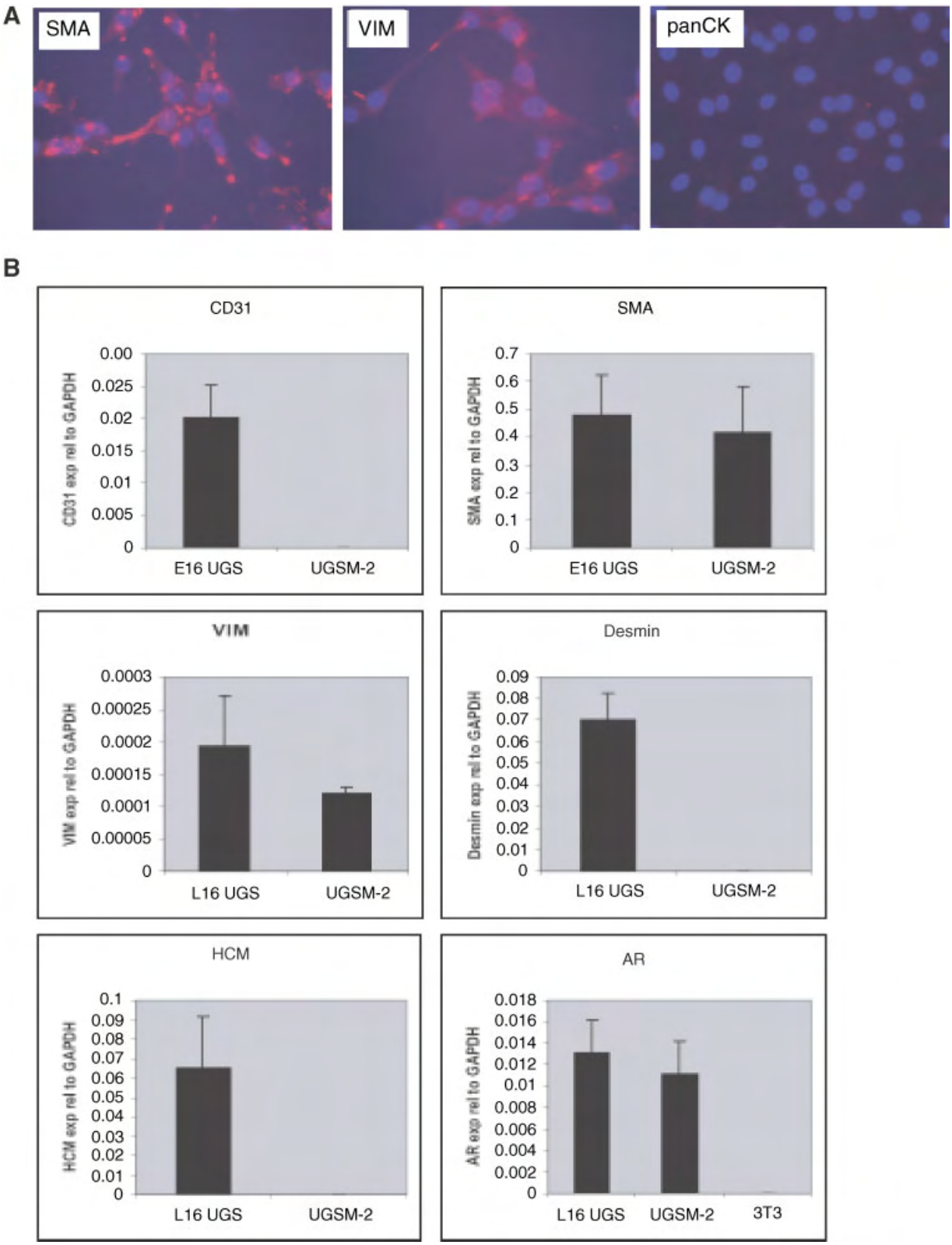

Fig. 2. (**A**) UGSM-2 cells immunostained for smooth muscle actin (SMA), vimentin (VIM), and high molecular weight cytokeratin (panCK). (**B**) Real-time RT-PCR analysis of expression of stromal differentiation markers by UGSM-2 cells and freshly isolated intact EI6 urogenital sinus (UGS). Genes studied include the endothelial marker CD3I, SMA, VIM, desmin, heavy chain myosin (HCM) and androgen receptor (AR). Each bar represents the mean +/− sem of at least 2 independent determinations.

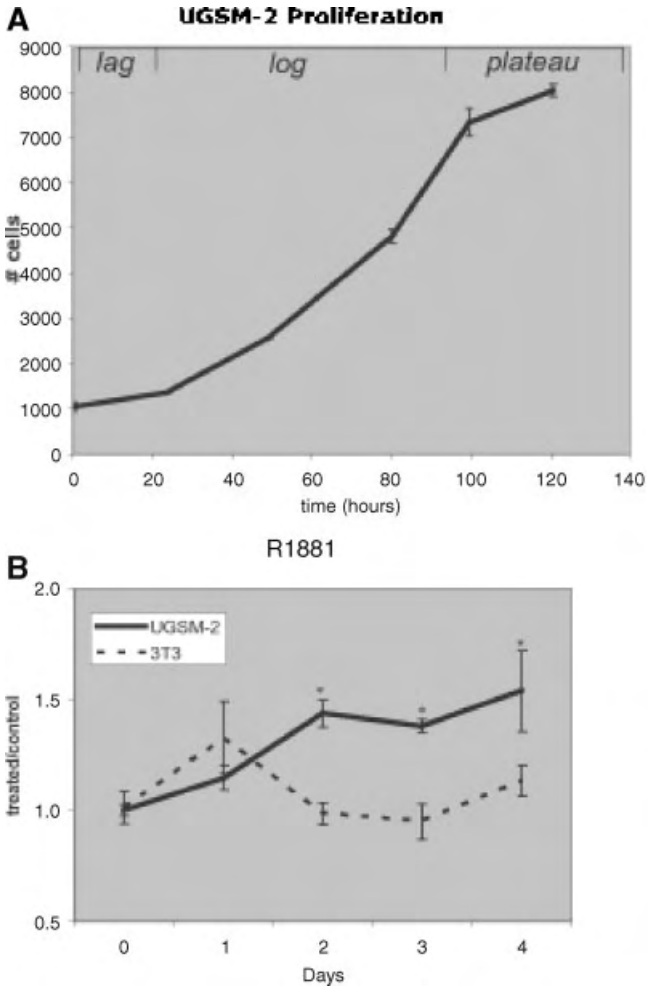

Fig. 3. Unique characteristics of UGSM cells. (**A**) Growth of UGSM-2 cells in culture. UGSM-2 cells were grown in normal INK4 medium (see Methods). Cell growth was monitored by daily Coulter counts for 1 week. Growth in culture exhibits three phases: lag, log and plateau. (**B**) Growth of UGSM-2 cells is stimulated by androgen. UGSM-2 cells were grown in charcoal-stripped serum media alone or with 10 nM synthetic androgen R1881. Cell growth was monitored by daily Coulter counts for 1 week. Data are shown as fraction of viable cells relative to untreated controls. No significant differences in cell viability were noted by Trypan blue staining (not shown). *significant increase in cell number, $P < 0.05$. All data points represent the mean of at least three independent determinations.

attributes of E16 UGS mesenchyme (UGM): the capacity to induce prostatic differentiation in the UGS epithelium (UGE), the potential to form the stromal component of prostatic glands, and the ability to mimic the signaling interactions of urogenital sinus mesenchyme. To examine the ability of UGSM-2 cells to induce prostate morphogenesis we grafted UGSM-2 cells together with isolated E16 UGE sheets under the renal capsule of adult male nude mice. When retrieved 1 month later, the resulting grafts were much smaller than grafts composed of E16 UGE and E16 UGM (Fig. 4A) and histologic examination did not reveal any evidence of glandular morphogenesis (not shown). Therefore, UGSM-2 cells are unable to induce prostate development in this model system. To determine whether UGSM-2 cells can participate in glandular morphogenesis during prostate development, UGSM-2 cells were grafted with minced E16 UGS under the renal capsule of adult male nude mice. UGSM-2 cells were pre-labeled with BrdU to trace their fate in matured UGS/prostate. The fate of UGSM-2 cells was examined after 1, 2, and 3 weeks of growth in vivo. UGS + UGSM-2 grafts had a similar size, gross morphology (data not shown) and histology (Fig. 4B) to minced E16 UGS implanted alone. Immunohistochemical staining for BrdU showed that BrdU-labeled UGSM-2 cells were present within the periductal stroma of the mature prostate tissue (Fig. 4C). The BrdU staining in nuclei of UGSM-2 cells exhibited varying degrees of speckling that increased from 1–3 weeks (data not shown). This was interpreted as indicating active UGSM-2 proliferation during growth of the grafted tissue.

To assess the interaction of UGSM-2 cells with adult prostate epithelial cells, UGSM-2 cells were co-cultured with human prostate epithelial BPH-1 cells. After 24 hr in co-culture, BPH-1 cells became organized into tight clusters surrounded by elongated UGSM-2 cells (Fig. 5A). When UGSM-2 cells were grafted together with BPH-1 cells under the renal capsule of adult male nude mice and the grafts examined one month later, the BPH-1 cells were organized into clusters surrounded by stromal cells very similar to those observed in co-culture (Fig. 5B). Mitotic figures were common in clusters, indicating active cell proliferation. Since BPH-1 cells injected alone do not form viable grafts, these observations suggest that UGSM-2 cells and BPH-1 can participate in a rudimentary process of cellular organization and that allows BPH-1 cells to survive and proliferate.

Shh Response of UGSM-2

To determine if the UGSM-2 cell line could accurately model the mesenchymal response to Shh signaling, we assayed gene expression in UGSM-2 cells treated with Shh peptide. When treated in cell culture with purified Shh peptide, UGSM-2 cells show robust activation of the conserved Hh target genes Gli1, Ptc1 and Hip. In addition, the Shh target gene IGFBP6, recently found to be upregulated in the UGS mesenchyme in response to Shh, was also induced (Fig. 6A). The three-fold increase in IGFBP6 expression after treatment with Shh is comparable to the response of the isolated E16 UGS mesenchyme to Shh [31]. To determine whether UGSM-2 cells would respond to

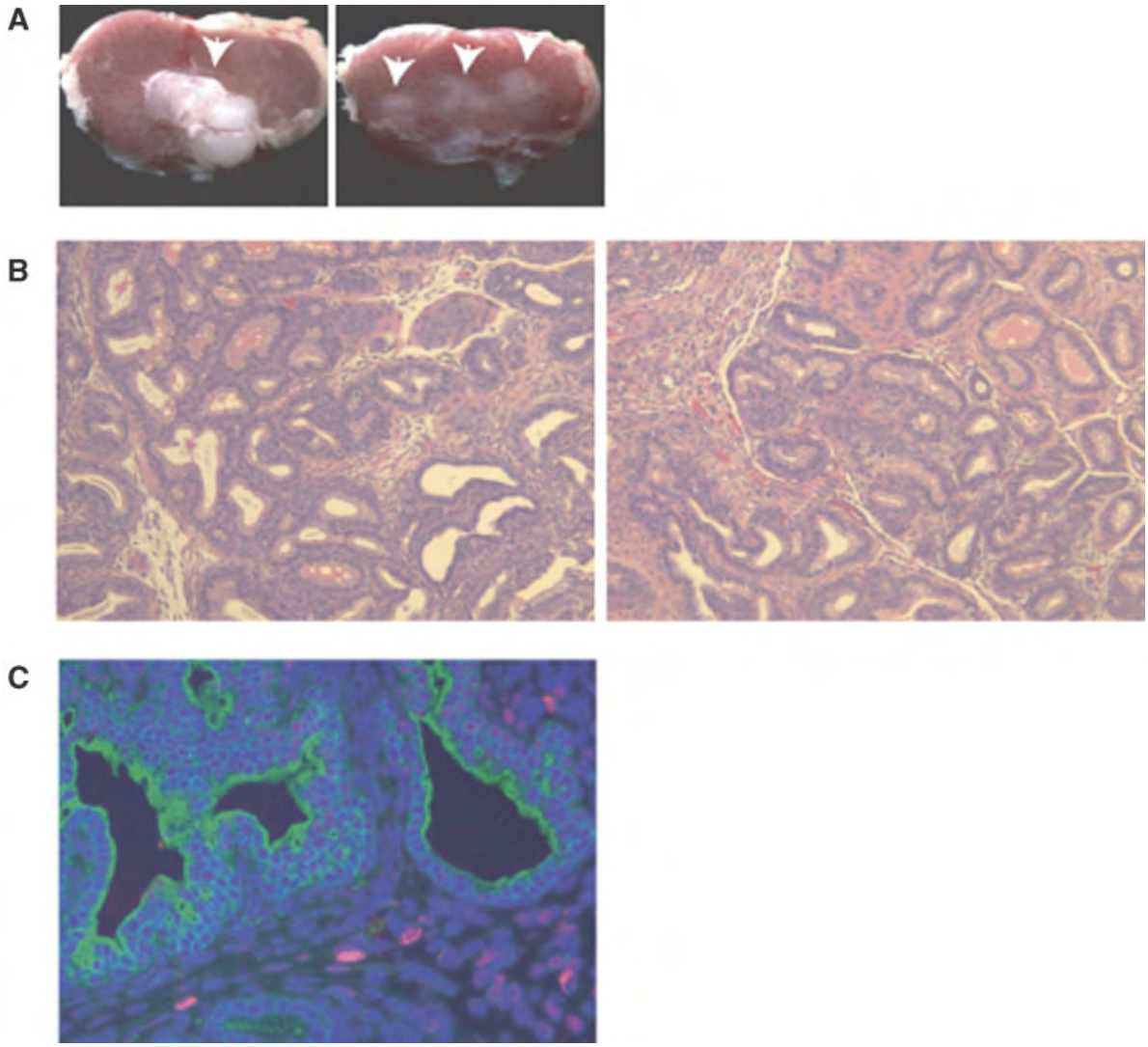

Fig. 4. Developmental fate of UGSM-2 cells in renal subcapsular grafts. (**A**) Gross morphology of 1 month renal grafts of E16 UGE combined with UGM (UGE+UGM, left) or UGSM-2 (UGE+UGSM2, right). Arrows indicate position of grafts. (**B**) H&E stained sections of UGS+UGM (left) or UGS+UGSM-2 (right) recombinants 2 weeks after grafting. (**C**) BrdU immunostaining (red) of 2 week UGS+UGSM2 renal grafts identifies UGSM-2 cells in the graft. DAPI stained nuclei are blue. Pan-cytokeratin identifies ductal epithelium in green.

Shh secreted by prostate epithelial cells in co-culture, we transfected BPH-1 cells with a Shh overexpression construct or GFP control vector (described in Fan et al., 2004). We cocultured UGSM-2 cells with the BPH-1 overexpressing or GFP control cells and analyzed Shh target gene expression using species-specific primers. This showed that overexpression of Shh by BPH-1 cells increased Gli1 and Ptc1 expression specifically in the UGSM-2 cells. There was no induction of Ptc and Gli1 in the BPH-1 cells (Fig. 6B). These experiments show that UGSM-2 cells in co-culture respond to a signaling ligand expressed by epithelial cells and can therefore mimic a stromal-epithelial interaction that plays an important role in prostate development.

CONCLUSIONS

Mechanistic studies of cell–cell interactions are facilitated by the use of genetically modified cell lines. Our long-term goal in developing the UGSM-2 cell line is to provide a tool for mechanistic studies of prostate development. We will use it to probe the mesenchymal signaling pathways that are important for prostate growth and differentiation. Urogenital sinus mesenchyme serves a critical role during prostate development as a medium for communication with developing epithelial glandular structures. Two of the signaling molecules involved in mesenchymal-epithelial communication during prostate development are androgen

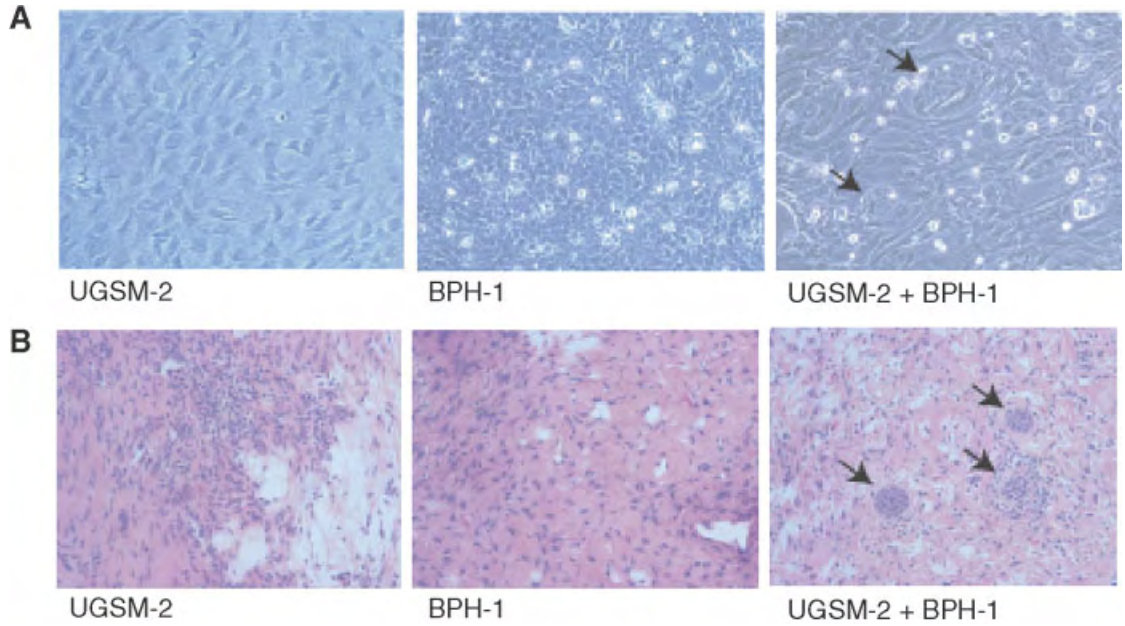

Fig. 5. Interaction of UGSM-2 and BPH-1 epithelial cells. (**A**) Photomicrograph of BPH-1, UGSM-2 co-cultures on collagen gels showing small clusters of BPH-1 cells (arrows) surrounded by UGSM-2 cells. (**B**) Grafting of UGSM-2 cells alone under the renal capsule yields only stromal tissue. BPH-1 cells grafted alone do not produce identifiable viable grafts. Grafting UGSM-2 and BPH-1 cells together results in small clusters of BPH-1 cells (arrows) surrounded by UGSM-2 cells.

and Sonic hedgehog. The ability of UGSM-2 cells to respond to both of these molecules makes it an appropriate tool for mechanistic studies of androgen and Sonic hedgehog activities in prostate development.

We found that these cells could not induce prostate differentiation when co-transplanted with the isolated sheets of E16 UGS epithelium tissue. However, we cannot exclude the potential of these cells to exhibit inductive potential in other assays such as one that uses dissociated UGS epithelial cells grafted under the renal capsule [32]. When UGSM-2 cells were mixed with and co-transplanted with the whole UGS, they clearly did populate the mesenchyme/stroma of the subcapsular graft. In these grafts, UGSM-2 cells took up various positions within the stroma of mature prostate. Some UGSM-2 cells were situated beside ductal epithelium, whereas others were embedded among other stromal cells in interductal stromal sheets. Although we have not analyzed stromal differentiation in these grafts, the ability of UGSM-2 cells to localize to different regions of the mature graft could indicate that they may take up both fibroblast and smooth muscle positions or functions in mature prostate tissue. Since UGSM-2 cells are able to occupy a stromal niche in developing UGS renal grafts, they may be used in in vivo gain and loss-of-function studies to examine the role of various gene products in early prostate development.

In addition to their ability to participate in prostate development, UGSM-2 cells form primitive acinar structures when either co-cultured or co-grafted with human BPH-1 prostate epithelial cells. Clustering of BPH-1 cells has been observed previously when co-cultured with primary fibroblasts derived from normal human prostate, but not with primary fibroblasts derived from human prostate tumors (Simon Hayward, personal communication). Cunha has shown that the inductive relationships between epithelium and mesenchyme are preserved between human and rodents [21]. Since the interactions between human epithelial cells and rodent mesenchymal cells are preserved, recombinants composed of human epithelium and UGSM-2 cells provide a useful model system for studying the role of these interactions in prostate development. An additional strength of this model is that we can distinguish signaling in mesenchyme and epithelium using species-specific RT-PCR. This dual species cell-based model therefore allows manipulation and analysis of gene expression in both epithelial and mesenchymal components to examine mesenchymal-epithelial interactions in vitro and in vivo.

In addition to their use in co-culture and xenograft models, UGSM-2 cells can be used as a cellular model to study mesenchymal signaling pathways that are important in prostate development. The first and most obvious use is to probe the molecular mechanisms of specific pathways. For example, we have used UGSM-2 cells to examine the concentration dependence and kinetics of Gli gene activation by Shh signaling (unpublished observations). The second is to use UGSM-2 cells in microarray studies to identify specific

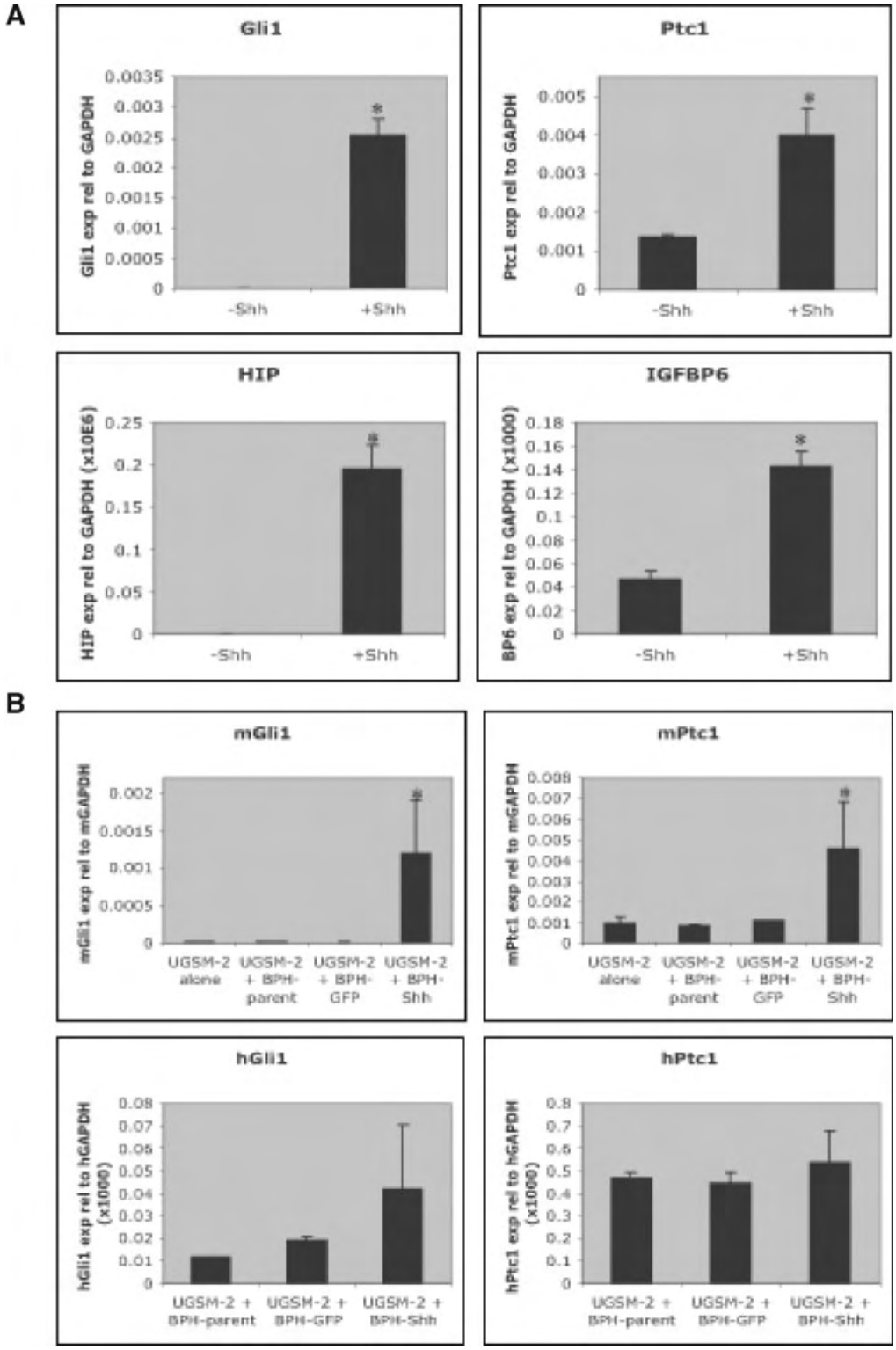

Fig. 6. Response of UGSM-2 to Shh. (**A**) Real-time RT-PCR of UGSM-2 cells treated with purified Shh peptide shows activation of Shh target genes Gli1, Ptc1, HIP and IGFBP-6. (**B**) UGSM-2 cells were co-cultured with BPH-1 prostate epithelial cells stably overexpressing Shh (BPH-Shh), empty vector (BPH-GFP) or untransfected (BPH-parent). RT-PCR analysis using species-specific primers shows significant activation of Shh target genes Gli1 and Ptc1 in mouse UGSM-2 cells (top), but not in human BPH-1 cells (bottom). *significantly increased from untreated or UGSM-2+BPH-GFP, $P = 0.06$. Error bar represents mean $+/-$ sem of at least 2 independent determinations.

target genes of selected inductive signals. Finally, the immortalized UGSM cells can be used for genetic gain- and loss-of-function studies. Overexpression of selected genes in UGSM-2 cells may be engineered to examine the gain-of-function effect. It should be noted that the INK4a mutant was created by insertion of a neomycin resistance gene and cell lines derived from this mouse are neomycin resistant.

Therefore, an alternative method of selection must be used when these cells are transfected. We have successfully used adenovirus, retrovirus, and plasmid vectors with hygromycin or zeocin resistance selection to express genes of interest in UGSM-2. UGSM cells are particularly useful in studying genetic changes that are lethal, since harvest of UGSM cells at E16 allows for isolation of cells even from non-viable mutants. Indeed, we have developed UGSM cell lines from INK4a$^{-/-}$ mice bred to transgenic lines with mutations in various Shh signaling pathway components.

The potential for immortalized stromal cell lines to become tumorigenic is well recognized. The INK4a$^{-/-}$ mutation produces impairment of G1 checkpoint control and the INK4a$^{-/-}$ mouse is prone to develop tumors in several mesenchymal tissues [28]. A recent report shows that INK4a$^{-/-}$ mouse embryonic fibroblasts display chromosomal rearrangements at high passage and develop the potential for sarcoma formation [29]. We have found that after 30 passages in culture, a mixed population of UGSM cells can form sarcomas when co-injected with Matrigel into nude mice. This can occur even while the cells remain contact inhibited and monolayer in culture (unpublished observations). However, we have never observed sarcoma formation with the UGSM-2 clonal cell line that was derived from the mixed UGSM population. Even so, we utilize the cells at low passage and perform sentinel grafts to monitor for sarcoma formation in all in vivo studies.

The UGSM-2 cell line and comparable cell lines derived from specific transgenic mutant mice will provide powerful tools to study signaling between prostate mesenchymal and epithelial cells. Using genetically modified UGSM cells in complementary cell-based assays, in vitro co-culture models and xenografts will allow detailed mechanistic studies of specific pathways and their influence on prostate development.

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Lack of Demonstrable Autocrine Hedgehog Signaling in Human Prostate Cancer Cell Lines

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Purpose: Several recent reports highlighted the role of hedgehog signaling in prostate cancer. However, the relative contributions of autocrine and paracrine hedgehog signaling to tumor growth and progression are unclear. Efforts to model autocrine signaling for drug development have been hampered by conflicting reports of the presence or absence of autocrine signaling in established human prostate cancer cell lines.

Materials and Methods: We comprehensively characterized the expression of hedgehog pathway genes in the 3 prostate cancer cell lines LNCaP, PC3 and 22RV1 (American Type Culture Collection, Manassas, Virginia). We also examined their response to Shh ligand and to the hedgehog pathway inhibitor cyclopamine (Toronto Research Chemicals, Toronto, Ontario, Canada).

Results: Expression of hedgehog ligand, patched and Gli1 in all 3 cell lines was lower than the expression level in normal human prostate tissue. All 3 cell lines showed hedgehog target gene activation when transfected with an activated form of Gli2 but none showed a detectable transcriptional response to hedgehog ligand or to transfection with an activated form of smoothened. Furthermore, treatment with the hedgehog pathway inhibitor cyclopamine did not inhibit hedgehog target gene expression in any of the 3 prostate cancer cell lines, although cyclopamine inhibited proliferation in culture.

Conclusions: LNCaP, PC3 and 22RV1 show no evidence of autocrine signaling by ligand dependent mechanisms and cyclopamine mediated inhibition of growth in culture occurs without of any discernible effect on canonical hedgehog pathway activity.

Key Words: prostate, prostatic neoplasms, growth and development, cyclopamine, gene expression

Hedgehog signaling is required for normal prostate development.[1–7] The Hh ligands Shh and Ihh are expressed in the epithelium of the urogenital sinus and the tips of the developing ducts. Expression of the Hh target genes Ptc and Gli1 primarily in the adjacent mesenchyma reflects a major component of paracrine signaling from epithelium to mesenchyma but focal expression of Ptc and Gli1 in the epithelium at the tips of the growing ducts has been interpreted as evidence of localized autocrine signaling.[6,8]

Several studies show active Hh signaling in human prostate cancer and provide evidence that Hh signaling accelerates tumor growth.[9–12] Xenograft studies show that paracrine Hh signaling alone can accelerate tumor growth. However, other studies suggest that autocrine signaling may also have a central role. Some studies suggest the operation of ligand dependent autocrine signaling, while others suggest the operation of ligand independent mechanisms of pathway activation resulting from mutation. The development of pharmacological inhibitors of Hh signaling for treating prostate cancer depends on further studies to define the relative contribution of autocrine and paracrine signaling in human prostate cancer, and the development of in vitro models for drug development and testing. Divergent reports of the presence or absence of autocrine signaling in several prostate cancer cell lines have slowed research and development. We report a comprehensive, mechanistic study of autocrine signaling in commonly used prostate cancer cell lines.

MATERIALS AND METHODS

Cell Lines

Prostate cancer cell lines were maintained in the recommended medium. BPH1 cells were grown in RPMI 1640 medium with 5% FCS. UGSM-2 cells[13] and MEFs were isolated at our laboratory. Four cDNA samples from independent human prostate epithelial cultures were used. Human prostate total RNA and fetal brain total RNA (BD™ Biosciences) were also used. Human prostate total RNA was pooled from the normal prostates of 32 white males at ages 21 to 50 years. Human fetal brain total RNA was obtained from normal fetal brains pooled from 21 spontaneously aborted male/female white fetuses at gestational ages 26 to 40 weeks. Cells were plated in a 24-well plate at 1×10^5 cells per well. To assay gene expression after Shh/cyclopamine treatment the serum concentration was decreased to 1% after 1 day attachment. Octylated N-Shh (Curis, Cambridge, Massachusetts) (1 or 10 nM) or 5 μM cyclopamine were added to the medium and RNA was harvested after 48

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hours. A 1 nM concentration of octylated N-Shh equates to a 400 nM dose of unmodified N-Shh.

Co-culture

UGSM-2 cells were plated at 1.6×10^5 cells per well in a 12-well plate. After 24 hours cancer cells were added on top of UGSM-2 cells at the same density. Cyclopamine (5 μM) or 1 nM octylated N-Shh was added to the medium and RNA was harvested after 24 hours.

Gli-luciferase Assay

Shh LIGHTII cells expressing Gli-responsive Firefly luciferase and TK-Renilla were plated in 10% fetal bovine serum at 90% confluence in Primaria multiwell plates and attached overnight. Medium was replaced with 1% fetal bovine serum with or without Shh peptide at given concentrations. After 48 hours Firefly and Renilla luciferase activity was assayed using a Dual Luciferase Assay System (Promega, Madison, Wisconsin).

Cell Proliferation Assay

Cells were set in a 24-well plate at a density of 20,000 cells per well and allowed to attach overnight. The concentration of FCS in the medium was changed to 2% and various concentrations of cyclopamine were added. Cells were grown for 4 days, harvested for RNA or trypsinized and counted by a Vi-CELL™ XR cell viability analyzer.

Adenovirus Infection

Adenovirus constructs carrying ΔNmGli2-GFP, hSmo*-GFP or GFP alone[14] were plated in a 24-well plate at 1×10^5 cells per well. After 24-hour attachment medium was replaced with 1% FCS with or without adenovirus at a multiplicity of infection of 25 to 100 pfu per cell with or without Shh peptide. Under these conditions greater than 90% of cells were infected according to GFP fluorescence analysis by flow cytometry.

RNA Isolation and Real-Time RT-PCR

RNA was isolated using QIAGEN® RNeasy® RNA isolation kits and subjected to on-column deoxyribonuclease digestion. cDNA was generated following standard protocols. Gene expression was assayed by real-time RT-PCR on an iCycler® instrument using GAPDH as an internal standard gene. The table lists the primer sequences used in this experiment.

Statistical Analysis

Each experiment was repeated 3 times independently. The unpaired t test was used to determine if statistically significant differences existed between treatment groups.

RESULTS

Hh Pathway Activity in Prostate Cancer Cell Lines

Comparison of Hh ligand expression in 4 prostate cancer cell lines showed that ligand expression was highest in PC3 and lowest in LNCaP (fig. 1, *A*). Shh and Ihh expression in PC3 was of the same order of magnitude as in the fetal brain but well below what was found in the normal adult prostate (fig. 1, *B*). Four primary epithelial cell lines isolated from human benign prostate tissue as well as BPH1 immortalized prostate epithelial cells showed expression that was intermediate between that of LNCaP and PC3 (fig. 1, *C*). Ptc and Gli1 are primary targets of Hh transcriptional activation. Ptc expression was highest in LNCaP and 22RV1, intermediate in PC3 and lowest in DU145 cells (fig. 2, *A*). Gli1 expression was similar in all cell lines (fig. 2, *A*). Ptc and Gli1 expression in these cell lines was generally comparable to expression in the 4 primary epithelial cell lines and BPH-1 but much lower than in normal prostate tissue (fig. 2, *B*). These studies revealed that the level of Hh ligand expression in all 4 cell lines was lower than that observed in pooled normal prostate specimens. Furthermore, pathway activity in the 4 cell lines, as judged by Ptc and Gli1 expression, was also considerably lower than that observed in pooled normal prostate specimens. Together these data do not suggest increased Hh pathway activity in these cell lines.

We noted that Ptc and Gli1 expression in the cell lines did not track the level of endogenous Hh ligand expression, suggesting that target gene expression may not be linked to ligand dependent pathway activation. Therefore, we examined the responsiveness of the tumor cell lines to exogenous Hh ligand. Using 1 and 10 nM concentrations of octylated Shh peptide, which elicit 75% and 100% of the maximal induction of Gli-luciferase reporter activity in NIH 3T3 cells, respectively, we observed no detectable increase in the Ptc or Gli1 expression in any of the tumor cell lines tested (fig. 3, *A* and *B*, and data not shown). Since serum levels are known to affect Hh responsiveness in vitro (unpublished data), we treated cells with 1 nM Shh under a range of serum conditions. Shh (1 nM) was unable to induce Ptc or Gli1 expression under 10%, 1% or 0.1% FCS conditions (fig. 3, *C*). To verify Shh activity in the same assays we treated the uro-

Quantitive real-time RT-PCR primer sequences

Gene	Forward Primer	Reverse Primer
mGAPDH	AGCCTCGTCCCGTAGACAAAAT	CCGTGAGTGGAGTCATACTGGA
mPtc	CTCTGGAGCAGATTTCCAAGG	TGCCGCAGTTCTTTTGAATG
mGli1	GGAAGTCCTATTCACGCCTTGA	CAACCTTCTTGCTCACACATGTAAG
hGAPDH	CCACATCGCTCAGACACCAT	GCAACAATATCCACTTACCAGAGTTAA
hPTCH	CGCTGGGACTGCTCCAAGT	GAGTTGTTGCAGCGTTAAAGGAA
hGli1	AATGCTGCCATGGATGCTAGA	GAGTATCAGTAGGTGGGAAGTCCATAT
hGli2	AGCCAGGAGGGCTACCAC	CTAGGCCAAAGCCTGCTGTA
hGli3	ATCATTCAGAACCTTTCCCATAGC	TAGGGAGGTCAGCAAAGAACTCAT
hShH	AAGGACAAGTTGAACGCTTTGG	TCGGTCACCCGCAGTTTC
hIhH	CACCCCCAATTACAATCCAG	AGATAGCCAGCGAGTTCAGG
hSmo	ACCTATGCCTGGCACACTTC	GTGAGGACAAAGGGGAGTGA
hHiP	CATGTCGTCATGGAGGTGTC	TCACTCTGCGGATGTTTCTG
hFused	GAGGGTGTACAAGGGTCGAA	TGCAAATTCCTCAGCTCCTT
hSufu	CGGAGGGGAGAGACCATATT	CACTTGGCACTGACACCACT

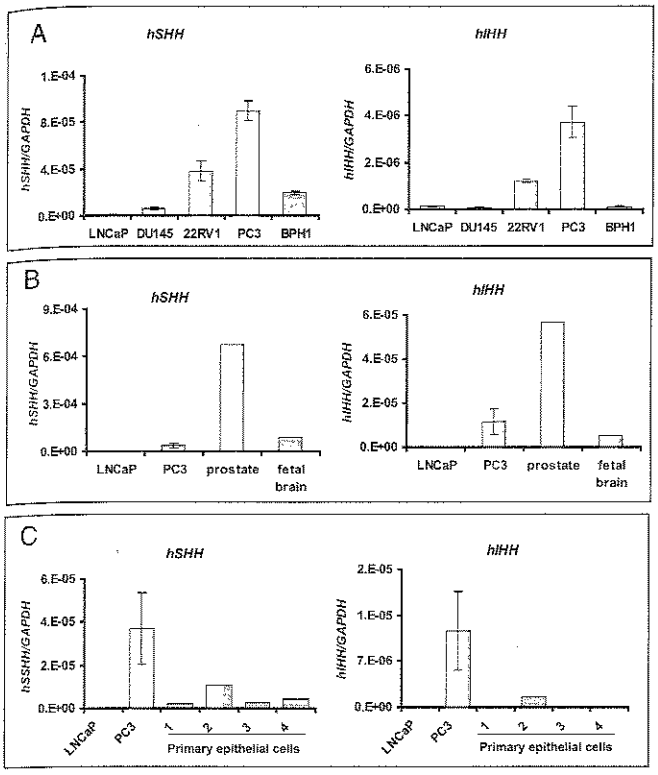

FIG. 1. *A*, Shh and Ihh expression in 4 prostate cancer cell lines LNCaP, DU145, PC3 and 22RV1, and normal human BPH-1 cell line. *B*, comparison of expression in LNCaP and PC3 with expression in human fetal brain and pooled sample of normal adult prostate RNA. *C*, comparison of expression in LNCaP and PC3 with expression in 4 primary benign prostate epithelial cell lines.

genital sinus mesenchyma cell line UGSM2 in medium containing 1% FCS with 1 nM Shh (fig. 3, *B* and *C*, inset). These observations are consistent with our previous observation that LNCaP stably over expressing Shh (LN-Shh) showed no evidence of pathway activation.[9]

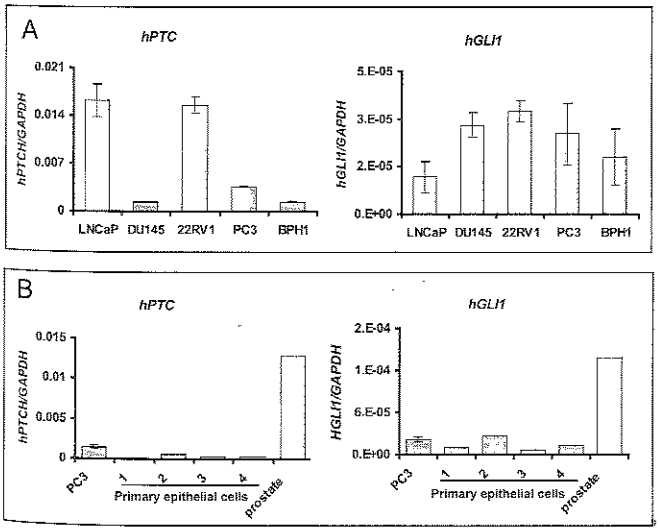

FIG. 2. *A*, expression of conserved Hh target genes Ptc and Gli1 in 4 prostate cancer cell lines LNCaP, DU145, PC3 and 22RV1, and normal human BPH-1 cell line. *B*, Ptc and Gli1 expression in PC3 and 4 primary benign prostate epithelial cell lines.

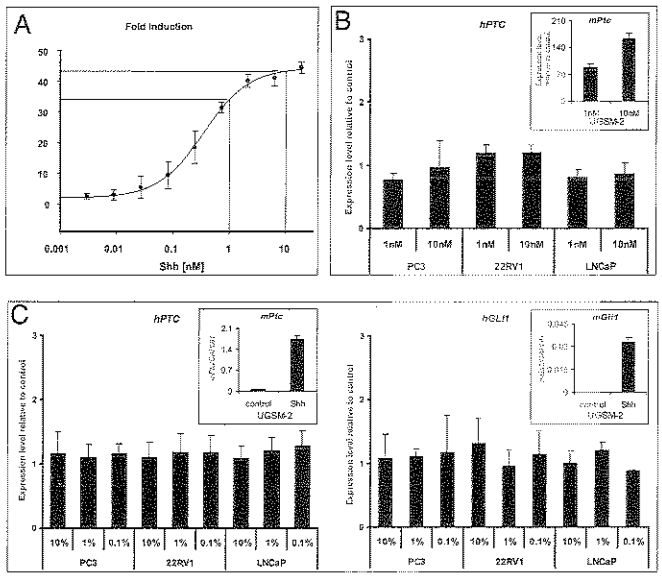

FIG. 3. *A*, dose response curve for Shh responsive Gli-luciferase reporter activity in NIH 3T3 cells (1 nM equals EC75 and 10 nM equals EC100). *B*, treatment of PC3, 22RV1 or LNCaP with 1 or 10 nM Shh did not increase Ptc expression. *C*, serum concentration did not alter Shh response of PC3, 22RV1 or LNCaP. Inset, 1 nM Shh was sufficient to significantly induce Ptc1 and Gli1 in UGSM-2 cells in 1% FCS ($p < 0.005$).

Intracellular Hh Signaling in Prostate Cancer Cell Lines

Each prostate cancer cell line expressed mRNA for the major components of the Hh signal transduction pathway, although the relative abundance of each factor showed considerable variation (fig. 4). Lack of responsiveness to Shh ligand could result from 3 mechanisms, including a block in ligand binding and transmembrane signal transduction, a defect in the intracellular signal transduction mechanism or a specific block in the transcription of Ptc and Gli1 in response to Hh pathway activation. To distinguish between these mechanisms we transiently expressed activated forms of Smo and Gli2 that have been shown to activate the expression of Hh target genes in many cell types.[9,15,16] The activated form of hSmo (Smo*) activates the intracellular signal transduction pathway and indirectly activates target gene transcription, whereas the activated form of mGli2 (ΔNmGli2) is considered a direct transcriptional activator of Hh target genes. Expression of Smo* in PC3 and 22RV1 cells did not induce Ptc and Gli1 expression in either cell line, whereas it induced robust Ptc and Gli1 expression in MEFs and UGSM-2 cells (fig. 5, *A*, inset and data not shown). In contrast, expression of ΔNmGli2 induced Hh target gene expression in the 2 cell lines. It induced robust expression of Ptc and Gli1 in 22RV1, and it induced robust expression of Gli1 but not Ptc in PC3 (fig. 5, *B*). The simplest explanation for the increase in Gli1 but not in Ptc expression in PC3 cells is that Gli1 is a more sensitive marker of induction because of its lower baseline level of expression. These studies suggest that the failure of PC3 and 22RV1 to respond to Hh ligand with Ptc and Gli1 induction results from a defect in the intracellular signal transduction mechanism in these cell lines.

Effect of Cyclopamine on Hh Signaling

The plant steroidal alkaloid cyclopamine inhibits Hh signaling by preventing Smo activation.[17] To examine endogenous,

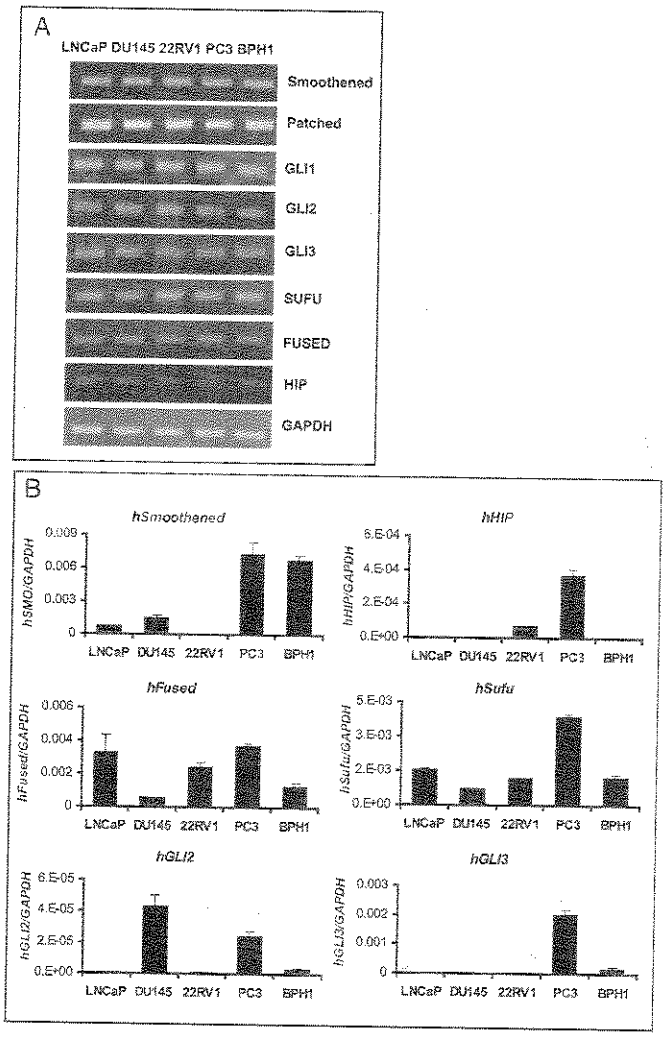

FIG. 4. Expression of Hh pathway genes Smo, Ptc1, Gli1, Gli2, Gli3, SuFu, Fused and Hip in LNCaP, DU145, PC3, 22RV1 and BPH-1. *A*, resolution of RT-PCR products after 40 cycles on 2% agarose gel using GAPDH as loading control. *B*, quantitative real-time RT-PCR for Hh pathway genes shows variations in steady state levels of individual pathway components.

Smo dependent Hh signaling in the cancer cells we examined the ability of 5 μM cyclopamine to block transcription of the Hh target genes Ptc and Gli1 in the cell lines. Regardless of whether the assay was performed in 10%, 1% or 0.1% FCS we observed no significant effect on Ptc or Gli1 expression in any prostate cancer cell line (fig. 6). We also observed no effect of cyclopamine when the assay was performed in the presence of 1 nM exogenous Shh peptide (data not shown). In contrast, 5 μM cyclopamine completely blocked Hh pathway activity in UGSM-2 cells stimulated with 1 nM Shh (fig. 6, inset). These findings, which demonstrated a lack of effect of the Smo antagonist cyclopamine, complement the lack of target gene activation by transfection with Smo* and further suggest the absence of Smo dependent autocrine signaling.

Effect of Cyclopamine on Tumor Cell Proliferation

Hh pathway activity has been implicated as a stimulus of prostate cancer cell proliferation, while inhibition of tumor cell proliferation in vitro by cyclopamine has been attributed to specific inhibition of the Hh pathway.[10–12] We examined the effect of cyclopamine on the growth of cancer cell lines in culture and correlated effects on proliferation with expression of the Hh target genes Ptc and Gli1. Treatment with 5 μM cyclopamine resulted in a decreased number of LNCaP cells after 4 days in culture, a slight decrease in the number of 22RV1 cells and no change in the number of PC3 cells (fig. 7). Treatment with 10 μM cyclopamine significantly decreased the number of cells after 4 days in all 3 tumor cell lines but this effect did not correlate with a significant inhibition of Hh pathway activity, as measured by Ptc and Gli1 expression (fig. 7, inset). These observations suggest that the inhibition of tumor cell proliferation in vitro by cyclopamine does not result from a specific effect on Hh pathway activity.

Cyclopamine was reported to inhibit the growth of PC3 tumor xenografts.[10] This was attributed to chemical inhibition of autocrine signaling in the xenograft. However, our studies did not demonstrate significant autocrine signaling in this cell line. To examine the possibility that cyclopamine might interfere with tumor growth by inhibiting Hh pathway activity in tumor stroma we examined the effect of cyclopamine on PC3 tumor cells grown in co-culture with UGSM-2 stromal cells. LNCaP cells over expressing Shh[9] were similarly co-cultured with UGSM-2 cells as a positive control for robust paracrine Hh pathway activation. Expression of the conserved Hh target genes Ptc and Gli1 was measured in human cancer cells and mouse stromal cells by real-time RT-PCR using species specific primers. Cyclopamine had no effect on hPtc and hGli1 transcription in the cancer cells themselves (fig. 8). In contrast, cyclopamine dramatically decreased mPtc and mGli1 transcription in UGSM-2 cells co-cultured with PC3 or LN-Shh cells (fig. 8).

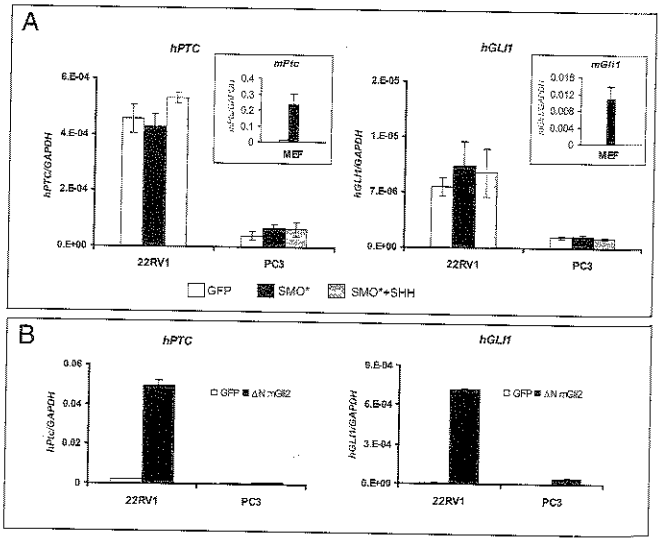

FIG. 5. *A*, infection of PC3 and 22RV1 with Smo* adenoviral vector did not activate expression of Hh target genes Ptc or Gli1 even when exogenous Shh was added. Inset, Ptc1 and Gli1 activation was achieved in MEF cells under same conditions ($p < 0.05$). *B*, infection of PC3 and 22RV1 with ΔNmGli2 adenoviral vector induced Hh target gene expression. PC3 and 22RV1 showed significant increases in Gli1 expression ($p < 0.05$). Ptc expression was significantly increased in 22RV1 but not in PC3 cells ($p < 0.005$ and 0.097, respectively). Adenovirus infection rates for all constructs was approximately 90%.

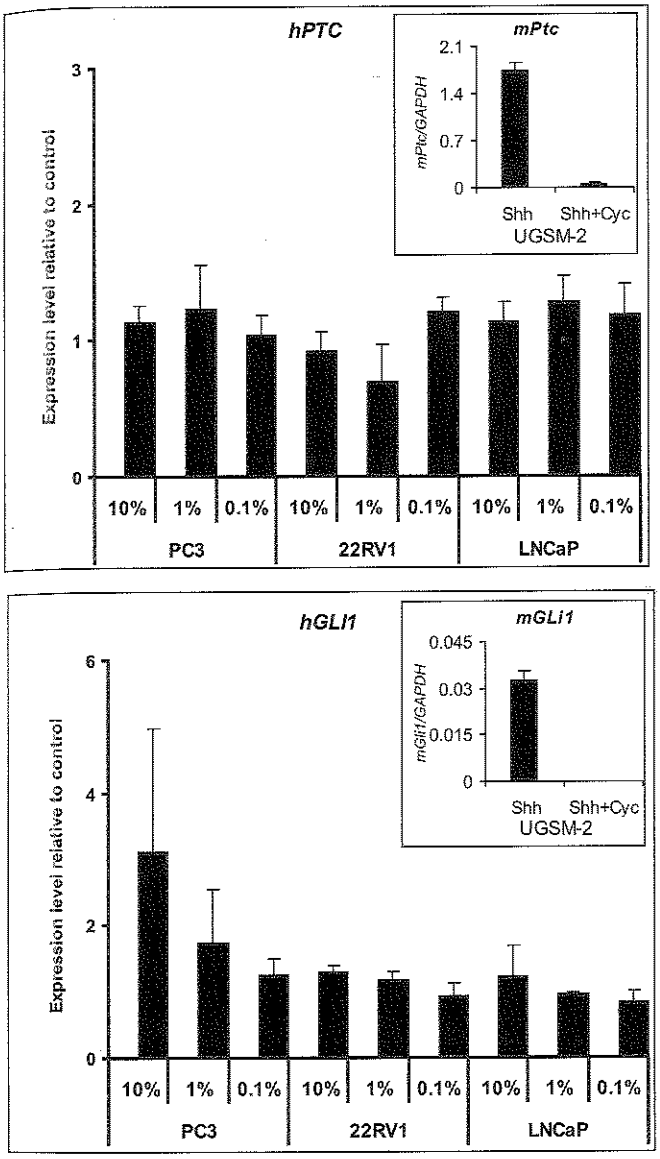

FIG. 6. Cyclopamine (5 μM) treatment of PC3, 22RV1 and LNCaP cells in medium supplemented with 10, 1 and 0.1% FCS did not alter expression of Hh target genes Ptc and Gli1. Inset, target gene expression was induced by Shh and inhibited by 5 μM cyclopamine in UGSM-2 cells ($p < 0.005$).

DISCUSSION

Our previous studies of Hh signaling in normal and neoplastic human prostate demonstrated comparable levels of expression of Hh ligand and Gli1 in specimens of benign and localized prostate cancer with a suggestion of higher level expression in locally advanced and/or androgen independent prostate cancer. We noted Shh expression in tumor epithelium with localization of Gli1 predominantly in periglandular tumor stroma and we used the LNCaP xenograft to determine that paracrine Shh signaling accelerates tumor growth.[11] Recently we observed that the paracrine effect of Shh signaling on tumor growth can be influenced by the composition of tumor stroma (unpublished data). Therefore, we speculate that Hh signaling may exert different growth effects in the normal prostate and in prostate cancer depending on the composition and/or reactivity of the stromal compartment.

Several other studies of Shh expression in localized and metastatic prostate cancer suggested that increased Shh expression in localized tumors exerts a combination of autocrine and paracrine signaling activity, and dramatically increases pathway activity in metastatic disease.[10–12] The possible contribution of autocrine signaling to tumor growth was examined by studying the effect of cyclopamine, anti-Shh antibody and Gli1 transfection on the proliferation of several human prostate cancer cell lines, including LNCaP, PC3 and 22RV1.[10–12,18] The studies suggest that these cell lines are characterized by high levels of Hh pathway activ-

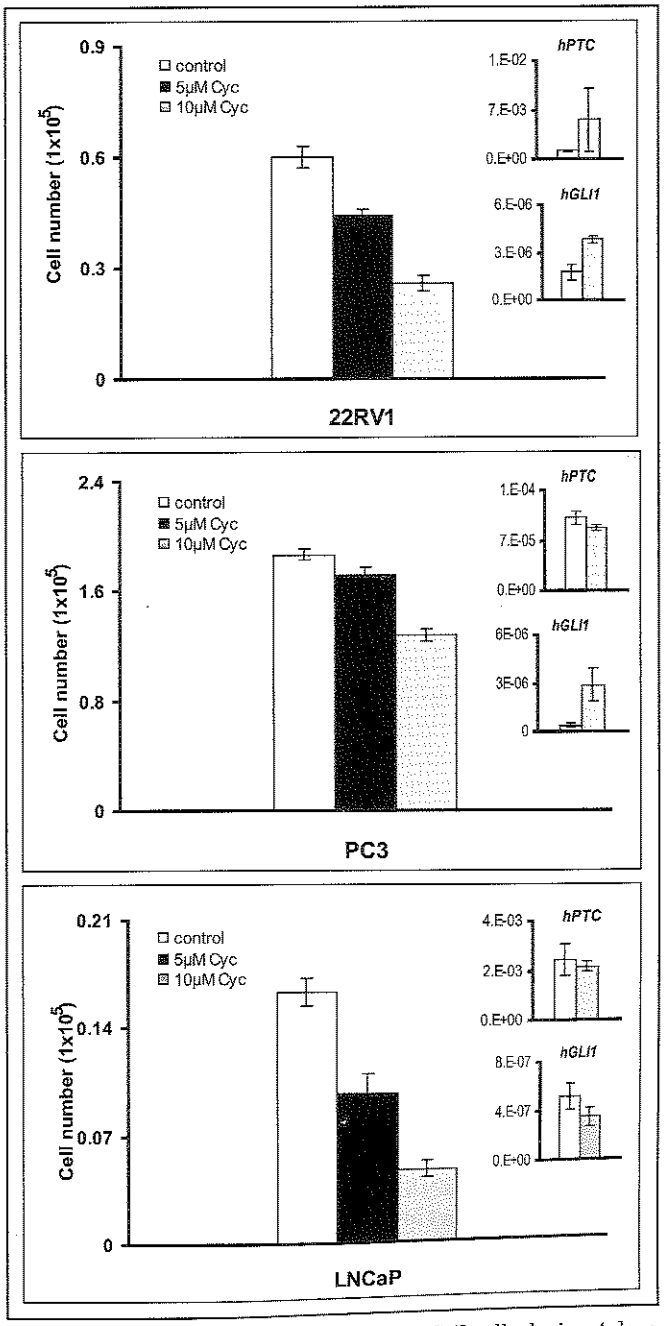

FIG. 7. Proliferation of 22RV1, PC3 and LNCaP cells during 4 days was inhibited by cyclopamine in dose dependent fashion (at 10 μM cyclopamine $p < 0.05$). Inset, in these cultures expression of Hh target genes Ptc and Gli1 was not altered by 10 μM cyclopamine, suggesting that decreased proliferation was not via Smo mediated event.

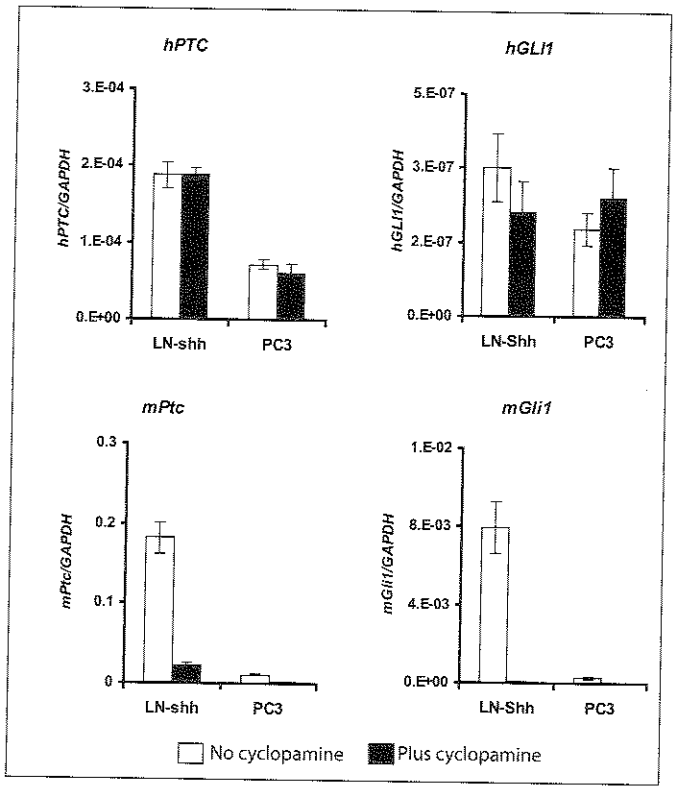

FIG. 8. Effect of 10 μM cyclopamine on autocrine and paracrine pathway activity in co-cultures of LNCaP cells over expressing Shh (LN-Shh) or PC3 cells co-cultured with mouse UGSM-2 cells. There was no effect on hPtc and hGli1 expression. However, inhibition of paracrine signaling in UGSM-2 co-cultured with LN-Shh or PC3 was evident from decrease in mPtc and mGli1 expression in presence of cyclopamine ($p < 0.05$).

ity, cyclopamine could inhibit tumor cell proliferation in culture by an Hh specific mechanism and cyclopamine could exert dose dependent inhibition of xenograft tumor growth. These studies clearly suggest that autocrine pathway activity promotes tumor cell proliferation and treatment with Hh inhibitors might be a promising avenue for therapy. However, these results are not entirely consistent. For example, Karhadkar et al found that anti-Shh blocking antibody inhibited PC3 proliferation,[10] whereas Sanchez et al found that PC3 proliferation was unaffected by anti-Shh blocking antibody or exogenous Shh.[11] Moreover, these findings conflict with our previous studies showing an absence of Hh pathway responsiveness in LNCaP.[9] For this reason we performed a comprehensive analysis of autocrine Hh pathway signaling in these cell lines.

Our studies show that LNCaP, DU145, PC3 and 22RV1 express Hh ligands and other components of Hh signal transduction. The level of ligand expression varied with the highest level of mRNA expression present in PC3 and comparable to the robust level of expression observed in the fetal brain. Even so, this was below the expression level in a pooled normal prostate sample composed of 32 prostate specimens from men 21 to 50 years old. The fact that expression was lower in all prostate cancer cell lines examined and in 4 primary prostate epithelial cell lines than in normal prostate is intriguing and it might suggest that in vitro culture conditions decrease Hh ligand expression. Similarly Ptc and Gli1 expression in these cell lines was much lower than in normal prostate, which might reflect a loss of autocrine signaling in vitro or signify that the primary domain of Ptc and Gli1 expression in the intact prostate is in the glandular stroma.

Since the tumor cell lines express the Hh ligands Shh and Ihh, pathway activity could result from ligand dependent autocrine pathway activation. However, our studies of LNCaP, PC3 and 22RV1 showed no evidence for a transcriptional response to exogenous Hh ligand. While the lack of response of LNCaP was consistent with our previous studies,[11] the unresponsiveness of PC3 and 22RV1 was unexpected and contradictory to previously reported studies. To validate these observations we examined the effect of intracellular pathway activation in PC3 and 22RV1 cells. Infection with an adenoviral vector expressing activated Smo did not induce Ptc or Gli1 transcription in either cell line. This observation argues that the canonical Smo mediated signal transduction pathway is nonfunctional. This was confirmed by the finding that transcriptional activation of the Hh target genes Ptc and Gli1 could be achieved in these cells by infection with an adenoviral vector expressing an activated form of Gli2 (ΔNmGli2). These studies, which demonstrate a nonfunctional post-receptor signal transduction pathway in PC3 and LNCaP, are consistent with a lack of responsiveness to Hh ligand.

Cyclopamine inhibits Hh signaling by binding to and preventing activation by Smo.[17] We observed no changes in expression of the Hh target genes Ptc and Gli1 in LNCaP, PC3 or 22RV1 treated with 5 μM cyclopamine under a range of culture conditions, a finding consistent with our transfection studies demonstrating a failure to induce Smo mediated Hh pathway activation. These observations stand in contrast to the studies of Karhadkar et al.[10] However, they examined the effect of cyclopamine on the expression of a Gli-reporter construct, rather than on the expression of endogenous Ptc and Gli1. It is possible that they observed an effect of cyclopamine on reporter gene expression that does not accurately reflect the effect of cyclopamine on the expression of endogenous target genes.

We observed that treatment of cells in culture with 10 μM cyclopamine decreased the cell number without any discernible effect on Hh pathway activity. These findings strongly suggest that inhibition of cell proliferation is not the result of canonical Smo mediated Hh pathway inhibition, but rather a nonspecific or toxic effect. However, how can we reconcile these observations with previously published studies showing a dramatic effect of cyclopamine on PC3 and 22RV1 xenograft tumors? An explanation is that PC3 and 22RV1 cells growing in vivo show a different phenotype and are susceptible to cyclopamine mediated inhibition of canonical pathway activity. Another explanation is that the effect of cyclopamine on xenograft tumor growth is mediated through an effect on stromal cells responding to Hh ligand produced by the tumor cells. This putative mechanism is supported by our co-culture studies and it suggests that the effect of Hh inhibitors on tumor growth may include effects on paracrine as well as on autocrine pathway activity.

Efforts are currently under way to develop Hh pathway inhibitors for clinical use. A critical step in this process is the development and use of appropriate cell lines and/or tumor models that depend on Hh signaling for growth. Based on previously published studies it has been assumed that human prostate cancer and commonly used prostate cancer cell

lines show robust autocrine signaling. However, our reported experiments revealed no evidence for autocrine Hh signaling in the most commonly used human prostate cancer cell lines under standard culture conditions and they showed no evidence that the Hh inhibitor cyclopamine could inhibit cell proliferation by a specific effect on Hh pathway activity. These findings caution against using these cell lines as an in vitro model of autocrine Hh signaling in prostate cancer. It is possible that the xenografts made with PC3 and 22RV1 might show autocrine signaling that cannot be modeled in cell culture but it is also likely that xenografts made with these Hh expressing cell lines also involve paracrine signaling interactions. Therefore, investigators testing the effect of Hh pathway inhibitors on prostate tumor xenografts should evaluate the effects of these agents on paracrine signaling as well as on autocrine pathway activity.

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Abbreviations and Acronyms

FCS	=	fetal calf serum
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
GFP	=	green fluorescent protein
h	=	human gene
Hh	=	hedgehog
Hip	=	hedgehog-interacting protein
Ihh	=	Indian hedgehog
m	=	mouse gene
MEF	=	mouse embryonic fibroblast
Ptc	=	patched
RT-PCR	=	reverse transcriptase-polymerase chain reaction
Shh	=	sonic hedgehog
Smo	=	smoothened
SuFu	=	suppressor of fused

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Hedgehog Signaling in the Prostate

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Purpose: Recent discoveries highlight the importance of the hedgehog signaling pathway in prostate growth regulation. We reviewed the role of hedgehog signaling in prostate development, adult prostate homeostasis and prostate cancer.
Materials and Methods: A comprehensive review of all relevant literature was done.
Results: Epithelial expression of hedgehog ligand during prostate development exerts autocrine and paracrine signaling activities that regulate growth and differentiation. Hedgehog signaling also occurs in the adult human prostate but to our knowledge the influence on epithelial proliferation and/or differentiation is unknown. Robust hedgehog signaling occurs frequently in prostate cancer, and autocrine and paracrine signaling have been shown to accelerate the growth of xenograft tumors. Autocrine signaling has been implicated in stimulating stem/progenitor cells and increased hedgehog pathway activity may be a characteristic of advanced, androgen independent cancer. The plant alkaloid cyclopamine is a specific chemical inhibitor of hedgehog signaling that produced sustained regression of established xenograft tumors.
Conclusions: Hedgehog signaling has an important role in prostate development and it appears to be a characteristic feature of prostate cancer. It stimulates tumor growth and may exert a specific role in the proliferation of tumor stem cells. The development of hedgehog inhibitors based on the action of cyclopamine holds promise for novel treatments to slow or arrest tumor growth.

Key Words: prostate, prostatic neoplasms, inflammation, ligands, growth and development

WHAT IS SO EXCITING ABOUT HH?

In a recent editorial comment in this journal Walsh described recent findings regarding the role of Hh signaling in PCa as being among the most important basic science findings related to PCa in the last 30 years.[1] Hh was first identified as an important signaling molecule in Drosophila. Hh signaling is conserved in vertebrates and it has an important role in fetal development of diverse structures, including the prostate gland. Recent study showed that Hh signaling promotes PCa growth and activated Hh signaling was identified as a key feature of clinically advanced disease. Even more exciting is the possible connection of Hh signaling to the proliferation of tumor stem cells, the small compartment of cells in a tumor that may be responsible for androgen independent tumor recurrence. Specific chemical inhibitors of Hh signaling have produced sustained regression of various xenograft tumors without overt toxicity to the adult host, suggesting that they may represent an entirely new class of therapeutic agents that could target previously untreatable cancers.

RELEVANCE OF DEVELOPMENTAL STUDIES TO CANCER

The Hh transcriptional activator Gli1 was first identified as an oncogene in glioblastoma.[2] Inactivating mutations in the Hh receptor Ptc were found in medulloblastoma[3] and in Gorlin/nevoid basal cell carcinoma syndrome.[4,5] More recently aberrant Hh signaling was found to be a consistent feature of various tumors originating in organs where Hh signaling has an important developmental role, including sporadic basal cell carcinoma of the skin, pancreatic cancer, small cell lung cancer, gastric cancer and PCa, prompting widespread speculation that reactivation of developmental signaling pathways is a critical step in tumor development.

A STEM CELL CONNECTION

An important facet of Hh signaling is its connection to stem cell proliferation.[6] Recent studies showed a role for Hh signaling in stem/progenitor cell proliferation in the central nervous system, mammary gland,[7] skin,[8] gut[9] and pancreas.[10,11] Hh signaling localizes to germinal cell populations in the developing central nervous system and it is required for the maintenance and expansion of progenitors.[12,13] Disruption of Hh signaling in the fetal brain decreases the number of neural progenitors, while Hh pathway activation in the mature brain increases the proliferation of telencephalic progenitors[12] and sustained pathway activation produces medulloblastoma.[14,15] These findings ignited speculation that Hh signaling is a key factor in sustaining proliferation of tumor stem cells.

A THERAPEUTIC OPPORTUNITY

Craniofacial birth defects in lambs born in Idaho in the 1950s were ultimately traced to the teratogenic effects of the alkaloid cyclopamine in the plant Veratrum californicum. The similarity to defects observed in the Shh null mouse[16] led to the discovery that cyclopamine is a specific chemical

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inhibitor of Hh signal transduction.[17] Cyclopamine has been used to examine the effect of Hh pathway inhibition on tumor growth and it showed dramatic treatment efficacy in animal models of basal cell carcinoma, medulloblastoma, pancreatic cancer and PCa. Recently topically applied cyclopamine showed remarkable efficacy against basal cell carcinoma of the skin in humans.[18]

OVERVIEW OF HH SIGNALING

Of the 3 mammalian Hh genes Shh, Ihh and Dhh, Shh is the most widely expressed during development. Shh binds to the specific receptor Ptc on the target cell surface and it activates an intracellular signal transduction pathway involving the Gli family of transcription factors. That activates the transcription of specific genes in the target cell (fig. 1).

Hh signaling is regulated at several levels. The transmembrane Ptc receptor constitutively represses Hh pathway activity through its interaction with a second transmembrane protein, Smo. Binding of Hh ligand to Ptc disrupts this interaction and de-represses pathway activity. Induction of Ptc expression by Hh signaling creates a negative feedback loop that re-asserts repression at the membrane level. A second mechanism for negative feedback is provided by Hh induced expression of Hip, a cell surface glycoprotein that sequesters Hh ligand. The 3 Gli genes Gli1, Gli2 and Gli3 encode transcriptional regulators that share a conserved DNA binding domain and bind the same 9 bp recognition sequence. Gli1 is a transcriptional activator of Hh target genes. Gli2 provides redundancy in the transcriptional activating functions of Gli1. Gli3 functions primarily as a transcriptional repressor that balances and refines transcriptional activation by Gli1 and Gli2. A third domain of Hh pathway regulation depends on a complex network of regulatory elements in the cytoplasm, involving protein kinase A and several other proteins, including Fused, SuFu and intraflagellar transport proteins, which regulate the location and activity of Gli proteins.[19]

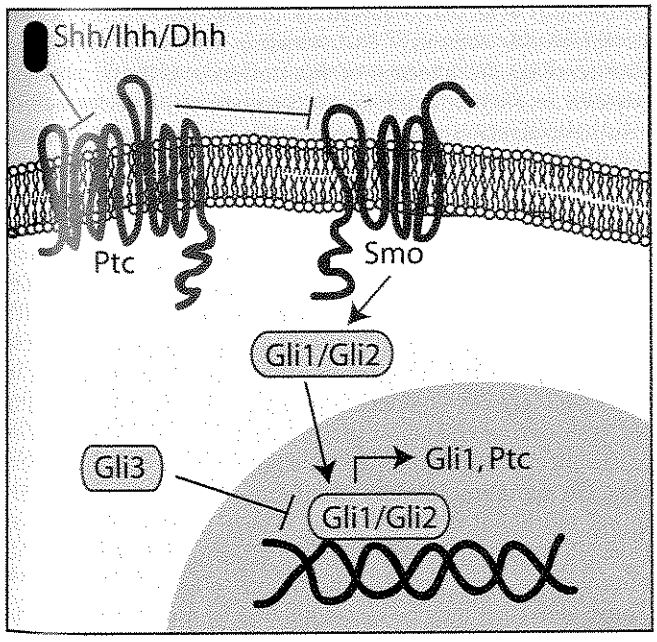

FIG. 1. Mammalian Hh signaling pathway. Hh ligands Shh, Ihh and Dhh bind to transmembrane receptor Ptc and relieve constitutive repression of Smo. Smo activation curtails transcriptional repression by Gli3 and promotes activation/translocation of Gli1 and Gli2 to nucleus, resulting in transcriptional activation of Hh target genes. Gli1 and Ptc are primary targets of Hh pathway activation and they serve as reliable indicators of Hh signaling.

HH SIGNALING IN PROSTATE DEVELOPMENT

During prostate ductal morphogenesis Shh expression localizes to sites of active growth. During ductal budding Shh expression in the epithelium is up-regulated and it condenses at sites of epithelial evagination. During ductal outgrowth Shh expression is strongest at the duct tip. Shh expression in the urogenital sinus does not depend on testosterone but testosterone modestly increases the level of expression and Shh redistribution during budding is certainly tied to an androgen induced morphogenetic event.[20] Blockade of Hh signaling by antibody blockade or chemical inhibition of Hh signaling disrupts ductal budding and glandular morphogenesis, respectively.[20,21] However, Berman[22] and Freestone[23] et al observed budding of the Shh transgenic null urogenital sinus and glandular morphogenesis in subcapsular renal grafts. The apparent discrepancy between these observations was resolved by our recent finding that Ihh provides functional redundancy for Shh. This conclusion is based on the observation that Shh null urogenital sinuses grown as renal grafts maintain expression of the Hh targets Gli1, Ptc1 and Hip, which correlates with increased Ihh expression in Shh null grafts.[24] Impairment of Hh signaling by transgenic Gli2 loss of function results in decreased Hh target gene expression, disruption of ductal budding, decreased expression of the stem cell marker Nestin and hyperplasia of $p63^+$ basal cells.[24] These studies show that Hh signaling and Gli mediated transactivation of Hh target genes are required for normal ductal budding and for balancing progenitor cell proliferation and differentiation.

Hh signaling can occur between tissue layers (paracrine signaling) or among cells in the same tissue layer (autocrine signaling). Ptc and Gli1, which are targets of Hh signaling, are tightly localized in the mesenchyma surrounding the nascent buds of the developing prostate. Localization of Shh expression to the tip of the elongating ducts is mirrored by Gli1 and Ptc expression in the surrounding mesenchyma.[20,25] Paracrine signaling directly affects mesenchymal proliferation[23,26] but it also influences epithelial proliferation and differentiation by paracrine feedback mechanisms.[20,26–28] In addition, there is concentrated epithelial expression of Ptc and Gli1, an indication of autocrine signaling, in nascent buds and at the tips of growing ducts.[25,29] Given that autocrine signaling stimulates progenitor cell proliferation in other organs, it is tempting to speculate that autocrine signaling at the tips of growing buds has a role in progenitor epithelial cell expansion (fig. 2).

Several recent observations are consistent with a role for Hh signaling in the maintenance and/or proliferation of prostatic progenitor cells. Gli2 loss of function and impaired Hh signaling are associated with decreased expression of the stem cell marker Nestin[24] in the prostate. Castration induced regression of the ventral prostate is associated with increased expression of Hh ligand, Smo and Gli1 (indicating increased Hh signaling), which is paralleled by increased Nestin expression. These changes are reversed during testosterone induced regrowth (unpublished data). Remarkably

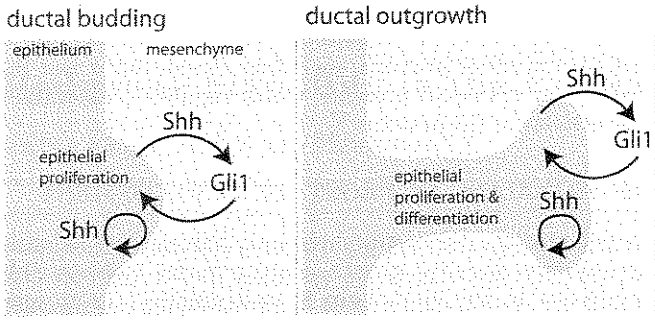

FIG. 2. Postulated actions of Hh in prostate development. *Left*, androgen dependent ductal budding is associated with Shh focal expression in epithelium of nascent buds. Shh acts on adjacent mesenchyma (*mesenchyme*) to activate expression of Hh target genes and it increases epithelial proliferation (paracrine signaling). Autocrine signaling at bud tip may stimulate progenitor cell proliferation. *Right*, during ductal morphogenesis autocrine signaling at duct tip stimulates continued progenitor cell proliferation, while paracrine signaling regulates epithelial differentiation.

chemical blockade of Hh signaling prevents testosterone induced regrowth.[30]

INFLAMMATION IN PCA

Chronic inflammation and oxidative stress were identified as key factors in predisposing to PCa development.[31] Indeed, lesions in the human prostate characterized by proliferating epithelial cells and activated inflammatory cells (proliferative inflammatory atrophy) are considered likely precursors of PIN and PCa.[32–34] An emerging paradigm postulates that epithelial injury and inflammation activate the proliferation of stem cells as part of the repair process. These proliferating progenitor cells are exposed to oncogenic forces, such as oxidative stress, that can induce genetic or epigenetic changes, leading to a persistent state of activation. The interaction between the persistently activated progenitor cell and the reactive stroma associated with inflammation and healing results in tumor formation and unregulated growth. The Hh and Wnt signaling pathways were identified as the 2 critical pathways regulating stem cell activation. In some tissues, such as the colon, activated Wnt signaling appears to be the dominant actor. In other tissues, such as the brain, skin, lung, pancreas and prostate, Hh signaling appears to have a key role in regulating stem cell activation and tumor development.[6]

Hh Signaling in the Developing and Adult Prostate

Shh is abundantly expressed in the human fetal prostate and it is down-regulated before birth.[27,35] Fan et al performed highly quantitative analysis of Hh signaling in the adult prostate using real-time RT-PCR to compare Shh, Gli1, Gli2 and Gli3 expression in normal prostate tissue from organ donors, BPH tissue obtained by prostatectomy, and tumor and zone homologous normal tissue from radical prostatectomy specimens.[27] The human fetal brain and fetal prostate were included as controls to compare the expression of these genes in tissues where the HH pathway activity is known to be high. These studies showed that Shh and Gli1 expression in specimens of normal prostate and BPH varied over several orders of magnitude but it was generally comparable to the robust level of expression observed in the fetal brain and fetal prostate. A tight correlation between Shh and Gli1 expression was observed, consistent with a dependence of Gli1 expression on Shh signaling. Karhadkar et al did not examine expression in the normal prostate per se but performed RT-PCR analysis for the presence or absence of Shh, Ihh and pathway gene expression in primary epithelial cells, benign prostate tissue adjacent to tumors, localized PCa and PCa metastases.[30] In their assays they observed that Shh and Ihh were expressed in primary epithelial cells, tumor associated benign tissue and localized PCa but the conserved Hh target genes Ptc and Gli1 were not. Ptc and Gli1 were only expressed in metastatic tumors. Sanchez et al examined normal human prostate tissue using real-time RT-PCR to compare the expression of Shh, Ptc and the Gli genes in 6 specimens of human PCa and tumor associated benign tissue.[36] These studies, combined with immunostaining of a tissue microarray containing tumor and tumor associated benign tissue, suggested a baseline level of Shh, Ptc and Gli1 expression in benign tissue, which is variably increased in tumor. Neither the Sanchez nor the Karhadkar[36] et al study included a reference control, such as fetal brain, to establish the relative level of expression in their specimens. This led to the widely shared perception that the level of expression of Shh and Ptc and Gli1 is low in benign prostate tissue but this interpretation is incorrect. The quantitative comparisons provided by Fan et al clearly show that Shh and Gli1 expression in normal adult and benign prostate tissues rivals the robust level of expression in the fetal brain.[27] This is reinforced by a recent comparison of expression in a pooled specimen of 30 normal prostate tissues and fetal brain showing high levels of expression of Shh, Ptc, Gli1 and Smo in the normal prostate.[37]

In situ hybridization studies using a highly specific, radiolabeled probe localized Shh expression to the prostatic epithelium and Gli1 expression almost exclusively to the periglandular stroma.[27] Ptc, which is expressed at a baseline level in the absence of Hh pathway activity, was expressed in each compartment. Sanchez et al performed in situ hybridization with a digoxigenin labeled probe and immunostaining to determine relatively weak co-expression of Shh, Ptc and Gli1 in the prostatic epithelium.[36] These studies suggest that Hh signaling in the normal/benign adult prostate may involve a combination of autocrine and paracrine signaling. While to our knowledge the role of Hh signaling in the adult prostate is not yet known, studies of Hh signaling during prostate development suggest a diverse repertoire of potential activities. Studies of Hh signaling in early prostate development highlight a role for Hh signaling for stimulating epithelial proliferation. In contrast, studies of the effect of Hh signaling in the postnatal prostate suggest that Hh signaling inhibits proliferation and stimulates terminal epithelial differentiation. These studies make clear that Hh signaling exerts multiple effects, including growth stimulatory and growth inhibitory effects.[20,22,23,25,26,29] These activities may be distinguished by autocrine vs paracrine signaling mechanisms and/or by an evolving response of mesenchyma to paracrine signaling as it differentiates. Whatever the case, it is clear that Hh signaling evokes various effects that might underpin homeostatic growth regulation in the normal adult prostate as well as in response to epithelial injury and inflammation.

HH SIGNALING IN PCA

Studies of Hh signaling in human PCa suggest that 1) autocrine and paracrine signaling contributes to tumor growth, 2) the effect of paracrine signaling may be influenced by the reactive character of tumor stroma, and 3) ligand dependent and ligand independent autocrine pathway activation is a feature of advanced disease. Fan et al compared Shh and Gli gene expression in tumor specimens obtained by radical prostatectomy to expression in specimens of BPH and normal prostate.[27] Mean expression in tumors was almost an order of magnitude higher than in benign specimens, although the difference was not statistically significant because of the wide range of expression in benign specimens. On separate analysis tumor and zone matched benign tissue from the same patients was examined, which showed generally comparable levels of robust Shh expression in the 2 tissues from the same patient. Karhadkar et al used RT-PCR analysis to compare Hh ligand expression and Hh pathway activity in specimens of localized and metastatic PCa.[30] They noted that Hh ligand was expressed abundantly in localized and metastatic PCa but, as evidenced by Ptc and Gli1 expression, Hh pathway activity was dramatically increased in metastatic lesions. They attributed this to the increased responsiveness to Hh ligand conferred by renewed Smo expression. An alternative explanation is that the Hh response in metastases is due to increased Hh sensitivity of stroma at metastatic sites. Sheng et al also observed an increase in Ptc expression in advanced PCa and they attributed some of the increase to mutations in SuFu, leading to dysregulated autocrine pathway activity.[38] Sanchez et al used RT-PCR analysis to find a variable increase in Shh expression and pathway activity in tumor tissue compared to matched benign tissue from the same specimen.[36] They used immunostaining for Shh to determine that increased Shh expression occurred in almost 33% of tumor specimens compared to less than 1% of benign tissues. Together these studies suggest that high levels of Shh and Gli1 expression are found in localized prostate tumors as well as in benign, zone homologous tissue in the same gland, and a further increase in Shh expression and Hh signaling occurs in advanced PCa.

Localization studies performed by Fan et al showed Shh expression in the tumor and glandular epithelium, and Gli1 expression primarily in the periductal stroma.[27] Sanchez et al performed in situ hybridization and immunostaining, and noted Shh, Ptc and Gli1 expression co-localizing to the tumor epithelium.[36] The apparent discrepancy in the location of Gli1 expression and, therefore, in the cell type showing pathway activation could be a product of different assay methods and/or might reflect heterogeneity of autocrine and paracrine signaling in PCa.

The commonly used PCa cell lines LNCaP, PC3, 22RV1 and DU145 express Shh and Ihh as well as the major components of the Hh pathway. The levels of expression vary considerably and the secretion of functional ligand has not been confirmed in most cases. Studies presented in 3 articles suggest that autocrine signaling in tumor cell lines stimulates cell proliferation, although there are significant discrepancies in findings at different laboratories.[30,36,38] Karhadkar et al found that anti-Shh blocking antibody inhibited PC3 proliferation in culture, suggesting that ligand dependent autocrine signaling stimulates cell proliferation.[30] However, Sanchez et al found that PC3 proliferation was unaffected by anti-Shh blocking antibody or recombinant Shh.[36] To our knowledge the discrepancy in these results has not been resolved. The Hh pathway inhibitor cyclopamine was found to inhibit the proliferation of PC3 and LNCaP cells in culture.[30,36,38] Cyclopamine inhibited Gli1 expression in LNCaP cells, arguing that the effect is pathway specific. However, the unresponsiveness of LNCaP cells to exogenous Shh[36] argues against operation of a ligand dependent pathway. The potential of chemical blockade of Hh signaling to inhibit tumor growth was examined by administering cyclopamine to mice with human PCa xenografts.[30] PC3 and 22RV1 tumors showed dose dependent inhibition of tumor growth, and complete and sustained regression at the highest dose tested. The specificity of this effect was confirmed by showing that xenografts made with tumor cells over expressing Gli1 were resistant to the antitumor effect of cyclopamine. These studies were interpreted as evidence that autocrine signaling in PC3 and 22RV1 tumors promotes tumor growth and can be inhibited by cyclopamine blockade. Additional experiments performed in rodent tumor cell lines showed that cyclopamine could inhibit growth and metastasis of the aggressive AT6.3 cell line, and Gli1 over expression conferred a highly aggressive and metastatic phenotype to the normally less aggressive AT2.1 cell line. While these observations are consistent with the notion that cyclopamine inhibits tumor growth by blocking Hh signaling, it is important to point out that the effect of cyclopamine on the growth of PC3, 22RV1 and AT6.3 tumors did not correlate with an inhibition of Hh signaling. Detailed studies under various conditions at our laboratory showed that LNCaP, PC3 and 22RV1 do not show the canonical transcriptional response to Hh ligand.[37] In addition, cyclopamine treatment did not produce an inhibition of Ptc and Gli1 expression even at concentrations that inhibited cell growth in culture. These observations, which are clearly at odds with previously published observations, were complemented by transfection based studies showing that the Hh signal transduction pathway is nonfunctional in PC3 and 22RV1. These findings are important for 3 reasons. 1) They show that PC3 and 22RV1 cannot be used to model ligand dependent autocrine signaling in human PCa. 2) They demonstrate that expression of Ptc and Gli1 in PC3 and 22RV1 is independent of the canonical Hh signal transduction mechanism and, therefore, it may be an inappropriate model in which to study ligand independent pathway activation that results from dysregulation of signal transduction. 3) These cell lines are not appropriate models for testing Hh pathway inhibitors based on the mechanism of action of cyclopamine.

The effect of paracrine signaling on tumor growth was examined using the LNCaP xenograft. Over expression of Shh by LNCaP tumor cells increased Ptc and Gli1 expression in tumor stroma without any evidence of autocrine pathway activation and accelerated tumor growth.[27] This suggests that Shh expressed by tumor cells acted on adjacent stromal cells to elicit paracrine signals that promoted tumor growth. Recently we observed that Hh pathway activation in tumor stroma alone is sufficient to accelerate tumor growth (unpublished data). Other recent studies show that the effect of tumor cell Shh expression on tumor growth is determined by the phenotype of the tumor stroma (unpublished observations). The dominant effect of the stromal

phenotype on the growth response to paracrine signaling may explain the differing effects of Hh expression in the growth quiescent normal prostate and in PCa, in which reactive stroma is generally present (fig. 3).[39]

HH SIGNALING, ANGIOGENESIS AND METASTASIS

Vascular endothelium is a well established target of Hh signaling. Shh induces the expression of pro-angiogenic molecules, including vascular endothelial growth factors and angiopoietins, by stromal cells. Vascular endothelial growth factors and angiopoietins stimulate endothelial proliferation and the growth of vessels into tumors. Hedgehog-interacting protein, an inhibitor of Hh signaling, is abundantly expressed in resting endothelial cells and it is down-regulated in PCa xenografts undergoing angiogenesis.[40] The pro-angiogenic effects of Hh may provide a growth stimulus for tumors and also a means to metastasize.

Hh signaling correlates with metastatic potential and Gli1 over expression can render a nonmetastatic cell line metastatic.[30] Hh signaling is implicated in mediating epithelial-mesenchymal transition, an event that is postulated to facilitate carcinoma invasion. Over expression of Gli1 in a nonmetastatic PCa cell line stimulated the expression of Snail, a marker of epithelial-mesenchymal transition, to levels seen in metastatic lines and it also increased cell invasion in vitro. In addition, Hh signaling may contribute to the predilection of PCa for bony metastasis since bone marrow stromal cells are responsive to Hh ligands, and Shh and Ihh stimulate bone remodeling.[41,42]

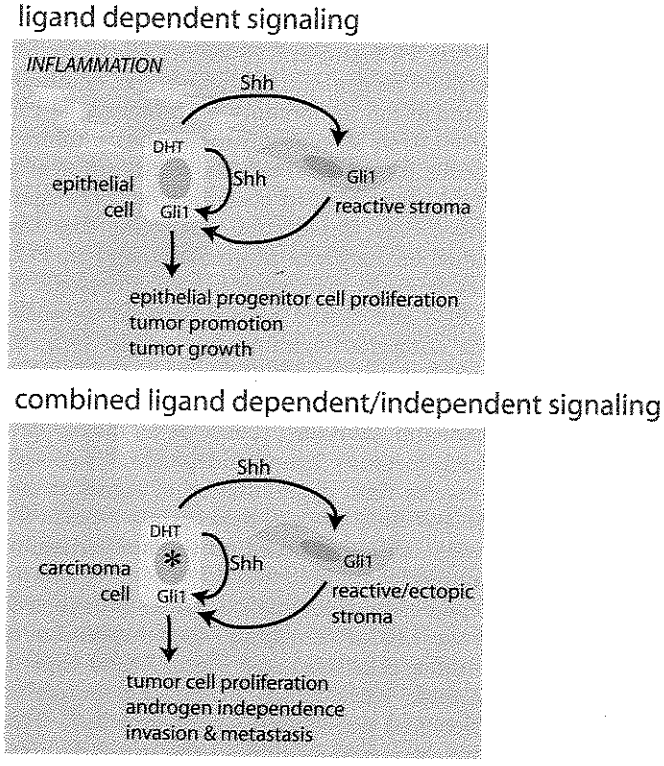

FIG. 3. Postulated actions of Hh in PCa. *Top*, injury and inflammation induce ligand dependent autocrine stem cell activation and proliferation, while paracrine signaling elicits growth stimulating responses from reactive stroma. This creates environment that promotes tumor formation growth. *Bottom*, tumor growth is accelerated by ligand dependent autocrine and paracrine signaling mechanisms, and by mutations (asterisk) that produce ligand independent pathway activation. These activities promote invasion, metastasis and androgen independent tumor growth.

CONCLUSIONS

Robust Hh signaling is characteristic of the adult human prostate and it may have various roles in homeostatic growth regulation and the response to injury or inflammation. Hh ligand expression and pathway activity are common in localized PCa and they may promote tumor cell proliferation by a combination of autocrine and paracrine signaling. Some of this may occur by canonical ligand dependent mechanisms and, as suggested by Sheng et al,[38] some may involve mutations affecting the regulation of Hh pathway activity in tumor cells. Hh pathway activity is dramatically increased in advanced metastatic PCa but to our knowledge whether this represents mutational activation or an increased responsiveness of the tumor cell or ectopic stroma to Hh ligand is not known.

Hh signaling is a unique target for therapy because of the apparently limited toxicity associated with chemical inhibition and the potential of this pathway to attack the postulated stem cell core of PCa. Recognizing that success in animal xenograft studies frequently does not translate to success in treating human cancers, what can we realistically expect? The first point to make is that Hh signaling occupies a unique niche in the signaling realm. There is some level of functional redundancy at the level of the ligand (Shh, Ihh and Dhh) and at the level of target gene regulation (Gli1, Gli2 and Gli3) but the signal transduction pathway appears to funnel specifically through the Ptc/Smo complex at the membrane level. There is little known cross-talk involving the Ptc/Smo receptor and, therefore, it is likely that inhibitors targeted to Ptc/Smo allows little room for escape by physiological mechanisms. Therefore, paracrine or autocrine signaling, which occurs by a ligand dependent mechanism, are promising targets for therapy. The stromal response involved in paracrine signaling is especially likely to depend on the canonical ligand dependent pathway and, therefore, it is a prime target for therapy to slow or arrest tumor progression. To our knowledge the relative contributions of ligand dependent autocrine signaling and mutational activation of the pathway to localized and metastatic tumor growth are as yet unknown. Autocrine signaling, which proceeds through an intact signal transduction pathway and regulatory mechanisms, is likely to be responsive to Hh blockade by cyclopamine analogues. However, autocrine pathway activity that occurs downstream from Ptc/Smo, for example through SuFu inactivation, can be expected to escape the action of cyclopamine-like inhibitors. Thus, it is possible that, as tumors progress and acquire an increasing number of mutations, they could acquire changes that result in autocrine pathway activation, which is unresponsive to Hh inhibitors based on the action of cyclopamine.

What is needed? The overly simplistic conclusion that Hh signaling is increased in PCa and tumor growth can be stopped by treatment with Hh inhibitors such as cyclopamine must be refined. We now know that Hh signaling is present in normal prostate as well as in cancer. To really understand what is going on we must understand how the roles of Hh signaling are similar and different in normal prostate and in PCa. This entails further studies to define

the relative abundance of autocrine and paracrine signaling in normal prostate, localized cancer and metastatic cancer, and mechanistic studies to examine how these activities are related to stem cell proliferation, amplifying or transit cell proliferation/differentiation and androgen regulation of growth and invasion. Moreover, we must identify what proportion of autocrine signaling in PCa is ligand dependent and what proportion results from intracellular pathway mutations. This information would enable us to select the tumor cell lines, xenograft models and animal models that most accurately represent human tumor and use them for drug development and testing. A novel and minimally toxic intervention that can cut to the root of a tumor is an exciting prospect for PCa treatment. Realization of the goal will require a great deal of study but it may not be so far away.

Abbreviations and Acronyms

Hh	=	hedgehog
Hip	=	hedgehog-interacting protein
Ihh	=	Indian hedgehog
PCa	=	prostate cancer
Ptc	=	patched
RT-PCR	=	reverse transcriptase-polymerase chain reaction
Shh	=	sonic hedgehog
Smo	=	smoothened
SuFu	=	suppressor of fused

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